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13. ABSTRACT ***(Maximum 200 Words)***
Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly new approaches are needed to treat these diseases. This project is designed to develop novel approaches to detect breast cancer cells that contaminate peripheral blood and bone marrow, and to remove such contaminating cells. An RT-PCR assay has been developed to detect breast cancer cells, and a novel gene therapy vector has been developed to kill contaminating cancer cells. Blood and bone marrow samples obtained from patients with breast cancer are being collected. These samples will be analyzed to determine whether the K19 RT-PCR assay can be used to predict outcome. Next, a gene therapy vector, the bcl-xs adenovirus, has been developed. This vector has promise as a therapeutic agent for the treatment of breast cancer. With additional support from the National Cancer Institute, this virus is undergoing toxicology testing in order to obtain FDA approval for human clinical trials for the treatment of breast cancer.

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INTRODUCTION

Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly new approaches are needed to treat these diseases.

One of the more promising approaches for the treatment of metastatic breast cancer is high dose chemotherapy. Breast cancer is susceptible to chemotherapy in a dose dependent manner. The major dose limiting toxicity of many effective chemotherapeutic agents is hematopoietic toxicity. To overcome this obstacle, autologous bone marrow or peripheral blood stem cells are harvested from the patient prior to the administration of the high dose chemotherapy, and then reinfused after the chemotherapy has been excreted and/or metabolized. Although the initial clinical trials of such strategies are promising, there are other obstacles that need to be overcome to optimize results. The agents used for the systemic treatment of this disease need to be improved. Additionally, bone marrow transplantation for solid tumors such as breast cancer is complicated by the fact that these cancers frequently metastasize to the bone marrow.

To date, two significant advances have resulted from this proposal. A sensitive assay has been developed to identify breast cancer cells in the bone marrow and the peripheral blood (Appendix, Manuscript #1). Next, and adenovirus vector has been developed that is selectively lethal to breast cancer cells, but not normal cells. The results of preclinical studies with this virus have been successful. The National Cancer Institute (NCI) has decided to hold an IND for this virus, and toxicology testing is underway in order to obtain FDA approval for clinical trials. This virus should enter clinical trials for the treatment of breast cancer sometime in the next two years.

BACKGROUND

The use of autologous bone marrow transplant (BMT) as a part of cancer treatment has increased dramatically in recent years. For example, autologous BMT often is used to protect patients from the hematopoietic toxicity of high dose chemotherapy in the treatment of breast tumors [1,2]. However, the success rate for this treatment regimen is jeopardized by contamination of the autologous marrow with low numbers of tumor cells [3-6]. Thus, the ability to selectively kill, or purge contaminating tumor cells in the marrow *in vitro* prior to autologous transplant could significantly improve the chances of long term survival.

The most common technique of purging tumor cells from bone marrow relies upon monoclonal "anti-tumor" antibodies to immunologically remove tumor cells from a suspension of marrow cells [7-9]. However, the success of this approach depends on the availability of well characterized antibodies capable of detecting each tumor cell phenotype. This method of purging rarely removes all tumor cells, and requires repeated purging cycles which can decrease hematopoietic cell viability [10]. Moreover, the specific antigens are rarely "tumor cell specific", often being found on normal cells, albeit at low

antigen density. Other purging methods, such as photo-sensitization agents or chemotherapy, also can cause significant hematopoietic toxicity [1,11,12].

Until recently, investigators thought that cancer treatments based on chemotherapy, or radiation therapy, exerted their tumor-killing specificities based on the different sensitivities of rapidly dividing (versus quiescent) cells to these agents. Recent evidence suggests that radiation and many chemotherapy agents specifically kill tumor cells, while sparing normal cells, by triggering of the Programmed Cell Death (PCD) pathway [13-15]. The induction of PCD is impeded by genes such as *bcl-2*, and its presence in cancer cells thus reduces the efficiency of conventional cancer therapy. Mechanistically, *bcl-2* does not appear to be mitogenic or transforming, but it cooperates with c-myc, and members of the ras family, to cause transformation [16-18]. Additionally, *bcl-2* acts to inhibit apoptosis induced by p53, *myc*, chemotherapy, and ionizing radiation [19-23].

Members of this gene family have been implicated in the progression of a large number of human solid tumors, including lymphomas, cancers of the breast, lung, and prostate, as well as neuroblastoma [24]. These observations suggest that suppression of *bcl-2* expression using gene therapy methods would be a valuable tool in cancer treatment, by increasing the susceptibility of tumor cells to existing chemotherapeutic and radiation treatments.

Other members of the *bcl-2* gene family have recently been isolated and partially characterized. A *bcl-2* homologue, *bcl-x*, gives rise to two mRNA species through alternative splicing. One of these, *bcl-*x_L, functions in a manner similar to *bcl-2*, and inhibits apoptosis. The other, *bcl-*x_S, functions as a repressor to *bcl-2* and acts to promote apoptosis. We recently demonstrated that adenoviral-mediated overexpression of *bcl-*x_S inhibited the anti-apoptotic role of *bcl-2* and induced PCD in a variety of primary tumors and tumor cell lines [25]. This PCD was augmented by, but not dependent upon, the tumor suppresser p53. In contrast, normal bone marrow hematopoietic stem cells resisted *bcl-*x_S-adenovirus induced PCD apoptosis. We postulated that the resistance of hematopoietic stem cells to the *bcl-*x_S adenovirus was due to the lack of expression of adenovirus transgenes in hematopoietic stem cells or alternatively to the inability of *bcl-*x_S to induce cell death in these cells. We report that murine hematopoietic stem cells resist expression of an adenovirally transduced gene. Moreover, a *bcl-*x_S adenovirus works synergistically with a Herpes virus TK adenovirus to specifically purge tumor cells from *in vitro* hematopoietic cultures, with the preservation of transplantable stem cells. Thus, these results indicate that adenovirus vectors fail to transduce genes into early hematopoietic stem cells. Therefore, this suggests that adenovirus vectors encoding suicide genes such as *bcl-*x_S or Herpes virus TK would preferentially kill the contaminating tumor cells derived from epithelial tissues found in bone marrow cell populations, thus serving as an excellent means of marrow purging.

Adenoviruses have great potential as vectors for cancer gene therapy, especially in those strategies that rely on the delivery of lethal genes into malignant cells. In this scenario, some of the weaknesses of these vectors for use in treating inherited diseases are irrelevant. For example, the lack of a sustained expression of the therapetic gene is not an issue because the goal is to kill the cells. Moreover, a local stimulation of the immune system may help to erradicate the malignancy, provided the viruses are not completely blocked before they reach the tumor. However, the main problem of these strategies, especially for the treatment of solid tumors, is the poor distribution of the vector in the entire tumoral mass. The killing of malignant cells by targeted delivery of lethal genes

has been conceptually demostrated in many preclinical studies using different approaches (Roth and Cristiano, 1997; Dranoff, 1998). Despite all these evidences, the general belief is that in order to achieve a clinically significant benefit, some sort of amplification of the initial effect is required (Han, 1998). Immunotherapy offers another possibility to do this, because once initiated, the immune response can be mantained and propagated (Rosemberg, 1999). However, new systems are in progress to amplify the intrinsic activity of gene therapy vectros.

When viruses are used as vehicles for gene therapy, they are modified in a way that they can infect and introduce their genome into the cells, but they cannot replicate. The modifications consist of the deletion of certain viral genes that are necessary for the replication of the virus. In the case of adenoviruses, there are five transcription units that are expressed before the onset of viral replication (Shenk, 1996). These are called early units (E1a, E1b, E2, E3 and E4). The E1a region encodes the first two polypetides that accumulate after viral infection. Apart from inducing the entry of the host cell into the S phase of the cell cycle, they have the ability to activate other transcription units in the viral genome (Lillie and Green, 1989). Therefore, without the E1a gene the virus is virtually unable to replicate. This unit is usually deleted in adenovirus-derived vectors, and the therapeutic cassete is introduced in its place. The E4 region comprises at least seven open reading frames whose functions are still not completely elucidated. The consequence of the deletion of this region is an inefficient production of viral particles due to deffects in viral replication, late gene expression, host cell shutoff and particle assembly (Halbert et al., 1985; Falgout and Ketner, 1987; Lusky et al, 1998). Interestingly, it has been described that the E4 orf 4 can induce p53-independent apoptosis (Marcellus et al., 1998; Shtrichman and Kleinberger, 1998).

A new generation of replication-conditional adenoviruses, also called tumor-specific replication-restricted adenoviruses (TRRA), has been recently developed to kill the malignant cells in a controlled fashion (Vile et al, 2000). The goal is to obtain viruses that can only replicate into the tumor. In this way, an initial dose of the therapeutic virus will infect a limited number of cells, but the natural cycle of the virus will continue in cancer cells, with the subsequent replication, death of the host cell and liberation of thousands of new viruses. Ideally, the process goes on until no more cancer cells are available for the virus to replicate, and it limits the spread of the virus to the tumoral margins. At least four different replication-conditional adenoviruses have been already developed, demostrating the feasibility and great potential of this new approach. In order to direct the replication of the virus to cancer cells, two different basic strategies have been used so far. The first one led to the construction of dl1520, an adenovirus that has a deletion in the E1b region (Bischoff et al., 1996). The E1b 55kd protein binds and inactivates p53 in the host cell, and this is necessary for its entry in the S phase of the cell cycle. Therefore, this virus can only replicate in cells with an abolished p53 function. Although this mechanism of action is actually controversial (Hall et al., 1999; Rothmann et al., 1998; Hay et al., 1999), the fact is that the results obtained with this virus, lately called 0NYX-015, are promising (Kirn et al., 1998; Heise et al., 1997; Heise et al., 1999). In a parallel way, a mutant adenovirus with a deletion in the E1a region has been described (Fueyo et al., 2000). The deletion unables the binding to the Rb protein, and hence limits the replication of the virus to cells with disrupted Rb pathway, which is a common characteristic of many cancer cells.

The second strategy for producing replication-restricted adenovirus consist of placing the E1 region under the control of tissue or tumor-specific promoters. In this way, prostate specific promoter and enhancer have been used to direct the viral replication to prostate cancer cells (Yu et al., 1999), and the α-fetoprotein gene promoter has been used to target the hepatocellular carcinoma cells (Hallenbeck et al., 1999). This method opens exciting posibilities for the treatment of many different malignancies, once a promoter is identified that is preferentially activated in the tumor. The search of such promoters can be optimized by using new technicques such as differential display (Peng and Vile, 1999) and serial analysis of gene expression (SAGE) (Velculescu et al., 1995), but the list of tumor-specific promoters is very limited. An alternative approach is the use of tissue-specific promoters to restrict the replication of the virus to the affected organ. For example, in up to 70% of breast cancers, the malignant cells retain the expression of Estrogen Receptors (ER) (Valavaara, 1997). In the presence of estrogens, these nuclear receptors can bind to a specific sequence (estrogen response element, ERE) present in the promoter of certain genes, and activate their transcription (Tsai and O'Malley, 1994).

A general characteristic of malignant tissues, especially solid tumors, is the hypoxic environment in which they grow. The development of a neoplasia requires the rapid generation of new blood vessels to supply oxygen and nutrients. The tumor vasculature is usually inefficient and it causes repeated periods of hypoxia and reoxigenation. Under these circunstances, the cells produce a transcription factor called hypoxia inducible factor (HIF), that activates the expression of certain genes after binding to hypoxia response elements (HREs) in their promoters (Bunn and Poyton, 1996; Maxwell et al., 1997). The HRE is a transcriptional enhancer that can be used to activate the expression of a gene in response to hypoxia (Dachs et al., 1997).

Significant progress in the purging of breast cancer cells has been made. We have further defined the use of adenovirus suicide vectors for killing breast cancer cells that contaminate the bone marrow of patients with breast cancer. We found that a pure population of mouse hematopoietic stem cells are not transduced by an adenovirus vector. We have extended this observation and now show that human hematopoietic stem cells are also not transduced by such vectors. The National Cancer Institute has now decided to do toxicology testing of the *bcl-xs* adenovirus to obtain FDA approval for use of the virus in human clinical trials. We envision such trials to begin within the next two years. In the last year of this grant, we have constructed and tested a new replication-conditional adenovirus for the treatment of breast cancer. We have included HREs in a promoter that contains EREs, and used this construct to replace both E1a and E4 promoters/enhancers in adenovirus type 5. We show evidence that this virus is more active in ER+ cells and can be used in combination with an E1a-deleted adenoviral vector to amplify the delivery of a pro-apoptotic gene to breast cancer cells.

BODY

Significant progress in completion of the goals of this grant has been made.

MATERIALS AND METHODS

Primary Bone Marrow Cells. Human bone marrow cells were obtained from the posterior iliac crest of normal volunteers following informed consent using a protocol approved by the University of Michigan Human Institutional Review Board. Bone marrow mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque (1.077 g/mL; Pharmacia). Cells were collected from the interface and washed three times in Iscove's modified Dulbecco's medium (IMDM; Gibco). Cells were then counted and divided for appropriate infection conditions. To isolate murine stem cells, bone marrow was harvested from the femur and tibia of c57black/Ka mice congenic for Thy 1.1. Bone marrow cells were stained with anti-Thy 1.1, anti-Sca-1, anti-Kit, and an anti-Lin cocktail consisting of anti-FcgII/FcgIIb, anti-Ly5.2, anti-CD3, anti-CD4, Anti-CD5, anti-CD8, anti-erythrocyte-specific antigen, anti-B220, anti-Gr-1, and anti-Mac-1[26]. Hematopoietic stem cells were isolated by FACS sorting twice as previously described (26). Reanalysis of the cells revealed greater than 95% of the sorted cells were Thy 1. 1^{lo}, Sca^{hi}, Kit^{hi}, Lin^{-}.

Adenoviral vectors. The *bcl-x_S* adenoviral vector, pAdRSV-bcl-x_S, was constructed by cloning a full length *bcl-x_S* cDNA into the pAdRSV vector [25]. This vector contains an RSV promoter and SV40 polyadenylation signal and allows high level expression of inserted sequences. Replication deficient virus was produced in the permissive human kidney 239 cell line containing complementary sub 360 sequences. Vectors were similarly constructed containing cDNA for thymidine kinase (TK)(pAdRSV-TK) or LacZ (pAdRSV-LacZ). Adenovirus infection and β-galactosidase assays were done essentially as previously described [25].

Hematopoietic Progenitor Cell Assays. Infection and viability assays were performed with adenoviral vectors as previously described [25]. For purging experiments, each infection condition utilized 1×10^6 hematopoietic cells admixed with 1.5×10^4 MCF-7 breast tumor cells that were stably expressing the G418 resistance gene. These cell mixtures were infected for four hours at a range of multiplicity of infection (MOI) from 2,000 to 10,000 viruses/cell in serum-free medium containing 1 ng/mL c-kit ligand and 10 ng/mL IL-3 (R&D, Minneapolis, MN). Following infection, cells were washed of virus and cultured for 48 hours in DMEM media containing 10% fetal calf serum, 10% horse serum, 0.1 U/mL Epo, 2 ng/mL IL-3, 5 ng/mL GM-CSF, and 10 ng/mL c-kit ligand. Cells were harvested and hematopoietic progenitor assays using 1×10^4 cells per assay were performed in triplicate as previously described [25]. Adenoviral infection of MCF-7 cells was assayed by determining colony development in the presence of 1 mg/mL geneticin (BRL/Gibco, Grand Island, NY) to kill normal hematopoietic cells. After two weeks, developing colonies were stained, scored, and photographed as previously described [25].

Murine Bone Marrow Transplants. All mice (C57bl/6, approximately 25 g) were purchased from Charles River (Wilmington, MA), and were used one week after arrival. Marrow cells for transplantation were obtained from the femora of male mice. Donor bone marrow cells from 4 male mice were pooled to yield a total of ~200×10^6 unfractionated marrow cells, which was then equally divided into the following groups for treatment with adenoviral vectors containing TK, Bcl-x_S, LacZ, or mock (identical treatment of marrow with adenoviral free reagents), as well as a group treated with TK containing vector followed by gancyclovir. Treated cells were transplanted into recipient

female mice (four per condition) irradiated with two doses of γ-irradiation (6 and 5 Gray delivered at 0.134 Gray/min) separated by three hours in order to decrease gastrointestinal tract toxicity. Bone marrow cells were transplanted by injections into either the tail vein or the retro-orbital sinus of anesthetized mice. Survival of the recipients was monitored for up to six months to determine long-term reconstitution.

Mouse Y-specific Sequence PCR Detection. Engraftment of transplanted male marrow cells was detected by PCR amplification of male-specific Y chromosome sequences in female recipients. Mouse Y specific PCR primers were synthesized with the sequences: Primer 1- 5' CAGTACCAGTCAGCAATATTTGTTG and Primer 2- 5' TTTCTGTATGCATTGTTTGTGAGT. DNA was extracted from bone marrow of recipient mice using a previously described method [27], and used as a template in the following PCR conditions: 200 mM Tris-HCl pH 8.8, 250 mM KCl, 35 mM $MgCl_2$, 200 nM each dNTPs, 250 nM each primer, 1μg template DNA, 2.5 U *Taq* Polymerase (Gibco/BRL, Grand Island, NY). Cycling parameters were 94°, 1 min; 55°, 1 min; 72°, 2 min; 25 cycles. The expected amplification product size is 316 bp. Reaction products were analyzed on a 1.5% agarose/TBE gel.

Detection of keratin 19 in blood and bone marrow.

Cell Lines, Patients, and Tissue Samples

Human mammary carcinoma cell lines T47D, MCF7 (estrogen receptor-positive), and SK3B3 (estrogen receptor-negative) were used in development of the RT-PCR assay. All three cell lines are positive for K19 by immunohistochemical staining. In addition, two mammary epithelial cell lines established from normal breast tissue obtained from women who underwent reduction mammoplasty were evaluated. To determine the sensitivity of the assay, peripheral-blood mononuclear (PBMN) cells obtained from a normal donor were mixed with decreasing numbers ofT47D cells. A total of 1 million cells were present in each sample. Cells were mixed before the RNA preparation thus mimicking the clinical setting for detection of mammary cells in the peripheral blood or bone marrow of patients.

The study population consisted of 34 patients with a histologic diagnosis of breast cancer in different stages, monitored at either the University of Michigan medical Center, Ann Arbor, MI, or at the Medical College of Wisconsin, Milwaukee, WI (Table 1). After approval by the institutional review board, and following informed consent, 2- to 5-mL samples of peripheral-blood or bone marrow aspirate were obtained from patients at various time points during treatment and processed as described later. Samples obtained from the peripheral blood of 10 non-breast cancer patients and bone marrow aspirates from 14 health, BMT donors and 15 patients following allogeneic BMT for chronic myelogenous leukemia (CML) served as negative controls. In addition, four patients with stage IV breast cancer underwent apheresis to obtain peripheral-blood stem cells before autologous BMT. Viably frozen aliquots of these cells were rapidly thawed, washed in phosphate-buffered saline (PBS), and RNA extracted as outlined later. Finally, 3 mL of CSWF was obtained from one patient with breast cancer who had carcinomatous meningitis documented by cytology. And from two additional patients without breast cancer.

Cell lines.

MCF7 (ATTC HTB 22) and T47D (ATTC HTB 133) are ER+ human breast cancer cell lines. MDAMB231 (ATTC HTB 26) is an ER- human breast cancer cell line. These cells were mantained in RPMI medium (BioWhittaker, Walkersville, MN)

supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 10 μg/ml insulin (GibcoBRL, Gran Island, NY). HeLa (ATTC CCL 2) is an ER- human cervical cancer cell line, and it was mantained in DMEM (GibcoBRL, Gran Island, NY) supplemented with 10% fetal bovine serum. When the experiment required depletion of estrogens, DMEM without phenol red was used (GibcoBRL, Gran Island, NY), supplemented with 2.5% charcoal-dextran stripped serum (Hyclone, Logan, UT). Estradiol (17βestradiol) and 4OH-Tamoxifen were purchased from Sigma (Sant Louis, MO). The 27-18 cell line (Gao et al) is derived from HEK293 cells after stable transfection with a plasmid expressing adenovirus E4 orf6 under the control of a MMTV promoter. These cells were mantained in DMEM plus 10% fetal bovine serum and 1 mg/ml G418 (GibcoBRL, Grand Island, NY). The expression of the E4 orf6 in these cells was induced by adding 10 μM Dexamethasone (Gensia, Irvine, CA) to the culture medium. All culture media were supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 μg/ml fungizone (BioWhittaker, Walkersville, MD).

Construction and characterization of the ERE/HRE promoter.

A cassette containing 3 copies of the HRE consensus (TGTCACGTCCTGCACGAC) flanked by Cla I sites was subcloned in the Cla I site of the plasmid pERE2pS2CAT (Montano et al., 1996), kindly provided by BS Katzenellenbogen, University of Illinois, Urbana, IL. This plasmid contains a portion of the pS2 promoter (-90/+10 bp), plus 2 of its EREs. The artificial promoter was liberated by digestion with EcoRI and BsrI, and subcloned in the SmaI site of the luciferase reporter plasmid pGL2Basic (Promega, Madison, WI). To verify the response to estrogens and hypoxia, the new construct (pBERE/HRE) was transfected into T47D, MCF7 or MDAMB231 cells using the Fugene 6 reagent, as described by the manufacturer (Roche, Indianapolis, IN). The cells (typically $7.5x10^4$/well in a 12-well plate) were seeded in estrogen-free medium and transfected 12-24 hours later using 0.5 μg of pBERE/HRE and 0,3 μg of a constitutive βgalactosidase reporter plasmid (pCDNA3βGal, kindly provided by G. Nunez, University of Michigan, Ann Arbor, MI) in order to quantitate the efficiency of tansfection. Twelve hours later, the transfection medium was removed and new medium was added containing the indicated treatments. After 24 hours, cells were lysed and analyzed for luciferase activity using the Luciferase Reporter Assay System (Promega, Madison, WI), as indicated by the manufacturer. The βgalactosidase activity was analyzed in cell lysates by adding the substrate O-Nitrophenyl-D-L-Galactopyranoside (ONPG), 0.9 mg/ml. The reaction buffer consisted of 1 mM $MgCl_2$ and 45 mM βMercaptoethanol in 100 mM sodium phosphate buffer. The samples were incubated at 37°C and the absorbance at 410 nm was quantified in an spectophotometer. The specific luciferase activity measured in a luminometer is presented as RLU (relative luciferase units), divided by the absorbance at 410 nm of the βgalactosidase assay.

Construction of Ad5HyERE.
Modification of the adenoviral genome.

In order to modify the regions containing the E1a and E4 promoters/enhancers, we subcloned portions of the adenovirus type 5 genome in intermediate plasmids that allowed their manipulation using standard cloning techniques. For the E4 region, the EcoRI-BamHI fragment of the pAdTrack plasmid (kindly provided by B. Vogelstein, Howard Huges Medical Institut, Baltimore, MD) containing nucleotides 34931 to 35935 of the viral genome (He et al., 1998), was subcloned into the pUC19 vector (New England BioLabs, Beverly, MA). The new plasmid was called pUC19Track. In order to eliminate the E4 promoter, a deletion comprising nucleotides 35575 to 35818 was obtained using the following strategy. The viral region comprising nucleotides 35233 to 35575 was amplified by PCR using primers that introduced a NruI site in the position 35575. The primers were 5' AAACTGGTCACCGTGATTAAAAAG 3' and 5' CCGTAGGATTCACCATCAGGTGTCGCGACGAGTGGTGTTTTTTAA 3'. The latter primer contains a 22 bp-long random sequence in the 5' end. In a similar way, the region comprising nucleotides 35818 to 35935 was amplified with primers that introduced a EclXI site in the position 35818, and produced a 22 bp sequence complementary to the previous one. These primers were 5' CACCTGATGGTGAATCCTACGGCGGCGGCCGACTCCGCCCTAAAACC 3' and 5' CGATCATTAATTAACATCATCAATAATAATATACC 3'. The products obtained wtith these sets of primers were combined to obtain a fragment in which the E4 promoter is substituted by the complementary random sequence flanked by NruI and EclXI sites. This fragment was amplified by PCR using the most external primers of the previous reactions, and was introduced in the $BstE_2$-PacI sites of pUC19Track. We then engineered an intermediate plasmid (pShutAd) containing the kanamycin resistance gene from pShuttle (He et al., 1998), the deleted E4 promoter region, and the E1a promoter-containing adenoviral sequence from nucleotides 1 to 1574 subcloned from the pTG3602 plasmid (Chartier et al., 1996), kindly provided by M. Mehtali, Transgene S.A., Strasbourg, France. Based on the pShutAd, we could introduce the ERE/HRE promoter into the E4 region, and started the modifications of the E1a enhancer and promoter. The deletion of the E1a promoter (nucleotides 341 to 499) was obtained by combination of two PCR fragments following the same strategy that led to the deletion of the E4 promoter. The fragment comprising nucleotides 341 to 499 was amplified with the primers 5' AGCGCGTAATATTGAATTCGGGCCGCGGGGACTTTGA 3' and 5' TCTACTCGCTGGCACTCAAGAGTCGCGACTTGAGGAACTCAC 3'. These primers incorporate EcoRI and NruI sites flanking the E1a promoter, and a complementary tail to the fragment produced in the second PCR reaction. The latter amplified the region comprising nucleotides 499 to 1339 The primers used were 5' CTCTTGAGTGCCAGCGAGTAGA 3' and 5' TGCATTCTCTAGACACAGGTGATGTC 3'. Once the two portions were fused and amplified, the product was subcloned in the SspI-XbaI sites of pShutAd, and the E1a promoter could be substituted by the ERE/HRE promoter. In the E1a enhancer/packaging signal, located upstream of the E1a promoter, point mutations were introduced that altered the enhancer consensus elements but left intact the packaging consensus (see sequence in figure 1A). Again, two complementary PCR products were produced, hybridized and amplified with the external primers to produce a fragment that was subcloned into pShutAd, in this case in the SgrAI-SspI sites. The primers for the first PCR fragment were 5' GTGTGCGCCGGTGTACACCATGCACATTCGTTTTCGCGCGG 3' and 5'

TCGATATTCAAATCTTACCCGGACCTCTACAAATTTACTACAACATCCGCC 3'. For the second fragments, the primers were 5' CCGGGTAAGATTTGAATATCGACGTTGGAAAACTGAATAAGGATTCGTGAAA TCTG 3' and 5' TAGACAAATATTACGCGCTATGAG 3'. Once the modifications of the E1 and E4 regions were completed, we used homologous recombination in E. Coli to introduce these changes in the whole adenovirus genome. The modified plasmid confers kanmycin resistance to the cells. It was cotransformed in BJ5183 cells with pTG3602, a plasmid that contains the wild type adenovirus genome subcloned in a backbone with the ampicillin resistance. After electroporation, the cells were selected in LB plates containing 30 μg/ml kanamycin and small colonies were screened by restriction endonuclease digestion. The DNA was purified from a single positive colony, and confirmed by automated sequencing of the modified regions.

Production and verification of the virus.

Once the plasmid containing the modified adenovirus was obtained, it was digested with PacI to liberate the viral genome from the plasmid backbone. After phenol/chloroform extraction and ethanol precipitation, the DNA was transfected into 27-18 cells using the lipofectamine method (Gibco BRL, Grand Island, NY), as described by the manufacturer. The cells were mantained in DMEM plus 5% FBS and 10 μM dexamethasone to induce the expression of the E4 orf 6. When the cytophatic effect was evident in the monolayer (typically 6-8 days after transfection), the cells were collected and lysed by three rounds of freezing and thawing in phosphate buffered saline (PBS) supplemented with 0.01% $CaCl_2$ and 0.01% $MgCl_2$ (PBS^{++}). After spinning the lysate at 350 g to discar the cellular debris, the supernatant was used to infect a monolayer of 27-18 cells. Viral plaques were isolated from this monolayer, and the structure of the virus was confirmed by PCR. The region containing the E1 promoter was amplified using the primers 5' TAGTGTGGCGGAAGTGTGATGTTG 3' (complementary to the beginning of the adenovirus sequence) and 5' TCTTCGGTAATAACACCTCCGTGG 3' (complementary to nucleotides 577 to 600). The region containing the E4 promoter was amplified using the primers 5' AAACTGGTCACCGTGATTAAAAAG 3' (complementary to nucleotides 35233 to 35251), and 5' CCGTAGGATTCACCATCAGGTGTCGCGACGAGTGGTGTTTTTTAA 3' (complementary to the end of the adenoviral genome). The PCR fragments were digested with ClaI, a restriction endonuclease site that is present in the ERE/HRE but not in this region of the wild type virus. Large scale preparation of the virus were produced in 27-18 cells and purified by cesium chloride centrifugation. Titrations were made by plaque forming assay, and multiplicity of infection (MOI) refers to plaque forming units (pfu)/number of cells.

Analysis of transcriptional activation of E1a and E4 units.

To analyze the responsiveness of the ERE/HRE promoter in the context of the adenoviral genome, we performed Northern blot assays of cells infected with Ad5HyERE. MCF7 cells ($2x10^6$ cells/60 mm plate) were pretreated for 12 hours with 17βestradiol, 4OH-Tamoxifen or $CoCl_2$ in estrogen-free medium. Infection was performed for 1 hour in 1 ml of PBS^{++}. The infection medium was then removed and the cells were incubated for 8 hours in the treatment medium. Alterantively, hypoxia was induced in the cells by incubating in an hypoxia chamber with 1% O_2, 6% CO_2. Hypoxia was induced 12 hours prior infection, cells were infected for 1 hour in normal conditions,

and then mantained under hypoxia for 8 additional hours. Total RNA was extracted using the Trizol reagent (Gibco BRL, Grand Island, NY), and 10 μg RNA were formaldehyde-formamide denaturated, fractionated in a 1.2% agarose gel with 2.2M formamide and transferred to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK). The E1a probe consisted of the 1 kb SspI-XbaI fragment from the adenoviral genome (nucleotides 341 to 1339), and the E4 probe was the 800 bp SspI-AsnI fragment (nucleotides 34634 to 35419). They were radiolabelled using the random primed DNA labeling kit (Roche, Indianapolis, IN). The membrane was prehybridized for 2 hours at 68°C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) in the presence of 1 mg/ml salmon sperm DNA and 1mg/ml tRNA. Hybridization was at 68°C for 1 hour with $2x10^6$ cpm/ml of the probe. The membrane was then washed with 2xSSC, 0.1% SDS at room temperature for 20 minutes, and twice with 0.1xSSC, 0.1% SDS at 50°C (1XSSC is 0.15 M NaCl and 15 mM sodium citrate , pH 7.0). Finally, it was autoradiographed. To confirm the homogeneous RNA lodading and hybridization, we used the same membranes and hybridized them with a mouse βactin probe.

Cytophatic effect and viability of cells.

After infection of the cell lines with a wild type adenovirus (Ad5WT) or Ad5HyERE, the appearance of cytophatic effect (rounding and dettachment) was monitored and cells were photographed using a digital camera (Pixera, Los Gatos, CA). To quantify the viability of cells, the MTT assay was used (Sigma, St. Louis, MO). At the time of analysis, 25 μl of 5 mg/ml Thiazolyl blue (MTT) solution were added to the cells cultured in 100 μl of medium (96-well plate). Five hours later, 100 μl of solubilization solution (20% w/v SDS in 50% v/v N, N-dimethylformamide) were added, and 12 hours later the absorbance at 600 nm was read in spectophotometer. The significance of the differences observed was analyzed using the *t* test.

X-Gal staining of cells in culture.

To analyze the complementation of the E1a defficiency of adenoviral vectors with Ad5HyERE, an E1a-deleted vector expressing the βgalactosidase (LacZ) gene (Ad5LacZ) was coinfected with Ad5HyERE. The cells infected with Ad5LacZ can be identified in the monolayer by fixing and staining with the substrate X-Gal (5-bromo-4chloro-3-indolyl-β-D-galactoside), that produces a blue precipitate upon hydrolysis by the enzyme βgalactosidase. The monolayers were fixed for 5 minutes in a solution containing 5.4% w/w formaldehyde and 0.8% w/w gluteraldehide in PBS. Then, the fixative was removed, the cells were covered by staining solution and incubated at 37°C until the blue precipitate appeared. The staining solution contained 1mg/ml X-Gal (GibcoBRL, Grand Island, NY), 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in PBS. The cells were then photographed, and the number of blue cells per field was counted.

Task 1. To test the hypothesis that women with poor prognostic indicators are more likely to present with the presence of blood and/or bone marrow micrometastases is ongoing with sample collections.

-1A. Sample collection and PCR assays. To date, we have collected blood samples from 123 patients including 20 patients with stage I or II cancer, nine with stage III , and 123 with stage IV breast cancer.

-1B. Clinical follow-up. Follow up of the outcome to the stage I, II and III prognosis patients is being monitored.

Task 2. To use a PCR based assay to detect the mammary cell specific keratin-19 mRNA and evaluate the presence of occult breast cancer cells in patients undergoing BMT.

PURPOSE: Detection of occult carcinoma in patients with breast cancer may aid the establishment of prognosis and development of new therapeutic approaches. To improve on existing methods of detection, we have developed a reverse-transcriptase polymerase chain reaction (RT-PCR) assay for keratin 19 (K19) transcripts to identify mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. **PATIENTS AND METHODS:** Peripheral-blood or bone marrow samples obtained from 34 patients with stages I to IV breast cancer and 39 control subjects without breast cancer were screened for K19 mRNA by nested primer PCR. **RESULTS:** In reconstitution experiments, K19 RT-PCR reliably detected 10 mammary carcinoma cells in 1 million normal peripheral-blood mononuclear (PBMN) cells. Four of 19 patients with stage IV breast cancer had detectable K19 transcript in peripheral blood. Five of six patients with histologically negative bone marrow biopsies following preablative chemotherapy and before autologous bone marrow transplant (BMT) were positive by this assay. Stem-cell apheresis harvests obtained from one of these patients and three additional patients immediately before BMT were all K19-negative. K19 RT-PCR analysis of CSF from a breast cancer patient with known carcinomatous meningitis was also positive. Thirty-eight of 39 non-breast cancer patients had negative K19 RT-PCR assays. The one exception was a patient with chronic myelogenous leukemia. **CONCLUSION:** RT-PCR of K19 is a sensitive, specific, and rapid method for detection of occult mammary carcinoma cells in the peripheral blood and bone marrow of patients with breast cancer. The presence of residual breast cancer cells in histologically normal bone marrow aspirates but not in stem-cell apheresis harvest is a frequent finding. This assay may be useful in diagnosing metastatic disease, as well as in monitoring the effectiveness of systemic therapy.

Two manuscripts have been published that address this task (see appendix).

-1A. To determine the relative frequency of tumor contamination of marrow versus peripheral blood stem cell harvests. To date, we have collected samples from 59 patients that have undergone BMT. Forty seven samples are from peripheral blood stem cell harvests, and 13 are from bone marrow harvests (one patient had both aphoresis and bone marrow). To date, 20% of the peripheral blood samples have been positive, and 46% of the bone marrow samples have been positive. At the time this grant was written, bone marrow harvest was the source of the hematopoietic cells used for rescue from high dose chemotherapy. Now, peripheral blood stem cell harvests are used exclusively for rescue from high dose chemotherapy. Since patients routinely get a diagnostic bone marrow prior to high dose chemotherapy, we have amended our protocol in order to analyze peripheral blood, the diagnostic bone marrow specimen, and the aphoresis product for K19.

-1B. Correlation of PCR results with clinical outcome. The positive rate in peripheral blood stem cell harvests is lower than that seen in bone marrow. Since peripheral blood stem cell harvests is used, only about 20% of the patients are K19 positive. Only about

20% of such samples are PCR positive for cancer. Therefore, the sample size is still too small to make any clinical correlation at this time.

-1C. Evaluate the efficacy of BM and stem cell culture purging techniques to eliminate breast cancer cells.

We have published a manuscript describes a novel method for purging contaminating cancer cells from bone marrow hematopoietic stem cells. Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x*$_S$, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating immune-deficient SCID mice were refractory to killing by the *bcl-x*$_S$ adenovirus. This vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation. (see manuscript in appendix).

We have previously shown that an adenovirus expressing *bcl-x*$_S$ can be used to selectively kill cancer cells that contaminate bone marrow (25). To better understand this observation, we determined whether murine stem cells express a transgene when infected with an adenovirus vector. To do this, Thy 1. 1^{lo}, Sca^{hi}, Kit^{hi}, Lin^{-} cells, which are the murine long term repopulating hematopoietic stem cell, were isolated from the bone marrow of c57black/KA mice by four-color FACS [26]. Either 1×10^3 hematopoietic stem cells or control neuroblastoma cells were exposed to 2×10^3-1×10^4 β-galactosidase adenoviruses/cell. As previously reported [25], neuroblastoma cells infected with even the lowest titer of virus expressed β-galactosidase. In contrast, hematopoietic stem cells infected with even 1×10^4 viruses/cell did not express β-galactosidase. These data suggest that adenovirus vectors based on the RSV Ad5 system do not transduce hematopoietic stem cells and that such viruses could be used to transduce suicide genes into tumor cells contaminating the bone marrow of patients undergoing high dose chemotherapy and autologous bone marrow transplantation.

In order to assess tumor purging effectiveness of the *bcl-x*$_S$ vector, *in vitro* cultures of human hematopoietic cells admixed with MCF-7 cells were treated with adenovirus. Our previous data indicated that an MOI of 2000 viral particles per cell was required to kill neuroblastoma cells. In sharp contrast, an MOI of 10,000 was required to completely kill all MCF-7 breast carcinoma cells. Cells from these mixed hematopoietic/MCF-7 cultures were then cultured in methylcellulose assays in order to assess hematopoietic progenitor cell survival. Numbers of CFU-GM colonies were used as a representative measure of progenitor survival. Some non-specific toxicity was noted in these cultures, as at a MOI of 2000, there was a slight decrease in CFU-GM numbers, whereas the CFU-GM colony number decrease was greater at a MOI of 10,000. However, this loss was non-specific as control vectors containing LacZ instead of *bcl-x*$_S$ caused similar reductions in CFU-GM numbers.

In an attempt to overcome the nonspecific toxicity of the *bcl-x*$_S$ adenovirus, a combination of *bcl-x*$_S$ and thymidine kinase (TK) containing adenoviral vectors was used in order to reduce the effective concentration of the *bcl-x*$_S$ vector. Expression of TK in infected cells leads to a sensitivity to the cytotoxic agent gancyclovir, which is added to the culture media after infection. Many cancer cells derived from cells of epithelial cells overexpress a member of the *bcl*-2 family. Since *bcl-2* can inhibit - and *bcl-x*$_S$ augment - chemotherapy induced apoptosis, a synergistic effect between TK and *bcl-x*$_S$ was

expected. Following a treatment with gancyclovir, tumor cell killing in TK/*bcl-x*$_S$ treated cultures was found to be as effective as the equivalent MOI of *bcl-x*$_S$ alone, but with reduced hematopoietic toxicity. Cultures treated with pAdRSV-*bcl-x*$_S$ at a MOI of 10,000 had CFU-GM numbers 26% less than cultures treated with a combination of pAdRSV-TK and pAdRSV-Bcl-x$_S$ at a MOI of 5000 of each vector. A colony replating assay revealed no detectable surviving MCF-7 tumor cells from these cultures (Figure 2B). Importantly, cultures of bone marrow cells mixed with tumor cells were purged equally effectively with either the *bcl-x*$_S$ or the *bcl-x*$_S$/TK treatment.

While the above *in vitro* assays indicated the ability of adenoviral vectors to selectively kill tumor cells, we wished to explore the effects of these vectors on the transplantable hematopoietic stem cell. To accomplish this, we used an *in vivo* murine models. The *in vitro* studies showing the inability of adenoviral vectors to directly infect the hematopoietic stem cell were confirmed by *in vivo* transplantation experiments. We determined the effects of adenoviral vectors on murine transplantable stem cells by their ability to reconstitute long-term hematopoiesis following lethal irradiation. Male bone marrow cells were treated *in vitro* at a MOI of 5000 particles per cell, and transplanted into female recipients. Importantly, marrow cells treated with adenoviral vectors containing either TK (with or without subsequent gancyclovir treatment) or *bcl-x*$_S$ rescued mice from lethal irradiation, indicating that transplantable stem cells were resistant to adenoviral infection. As expected, control mice receiving mock-infected marrow also survived, while mice receiving no transplant after irradiation had substantially reduced survival. Moreover, both short- (4 weeks) and long-term (six months) hematopoietic reconstitution occurred in these experiments indicating that both the more committed hematopoietic progenitor cells, as well as the hematopoietic stem cell are resistant to adenoviral infection (data not shown). Finally, the contribution of male donor cells to engraftment was confirmed by PCR detection of mouse Y sequences in the female recipients.. These data show that male-specific Y sequences are detected in marrow genomic DNA from all of the female survivors six months after transplant.

Task 3. Develop additional markers for molecular detection of occult breast carcinoma.

-3A. Evaluate the specificity and sensitivity of PCR based detection of other mammary specific RNA sequences. No new markers have been detected. The K19 marker has been both sensitive and specific. This made this a low priority task, and efforts were concentrated on the K19 effort and the adenovirus vector.

-3B. Develop non-radioactive detection schema. A nested primer approach was used to detect K19 cDNA (see appendix, manuscript #1). PCR experiments using K19 primers labeled with either 6FAM or HEX. The PCR products were analyzed with an automatic sequencer. Various amounts of MCF-7 breast cancer cell RNA was mixed with normal bone marrow RNA (from 1x 10^6 cells). These mixing experiments showed that after 2 rounds of PCR we were able to detect K19 mRNA in 2/3 samples containing 10 MCF-7 cells, and 2/4 samples containing 1 MCF-7 cell. K19 was not detected in normal peripheral blood. This task is completed, a non-radioactive detection schema has been developed. Either nested PCR with gel electrophoresis, or with fluorescent detection using an automated sequencer, can be used to detect the K19 mRNA.

-3C. Detection of novel breast cancer peptides. Breast cancer cDNA has been made from RNA isolated from SUM 159 human breast cancer cells. To date, no novel cell surface molecules have been identified in this library.

-3D. As in 3C, no new breast cancer specific peptides have been identified.

Since this grant was awarded, the gene therapy vector (bcl-xs adenovirus) has shown potential utility for the treatment of cancer. Therefore, an amended statement of work was approved by the DOD in order to develop animal models to test the potential clinical utility of this vector.

Task 4: In vivo delivery of adenovirus vectors. *In the final year of the grant, we wished to explore the delivery of our adenovirus vector in animals. We wished to explore delivery via intra-tumoral injection, as well as IV, intraperitoneal and arterial injection. In the extended year, we have made progress on this task. Realizing that a replication deficient virus could not be effectively delivered in vivo, we devised a system that allows selective replication within a tumor. We found that this system allows us to effectively cause regression of an established tumor. Time constraints have allowed us to only complete the intra-tumoral injection part of this task. We still intend to complete the remaining parts of this task, but it will be after the funding has ended. This work has resulted in a publication (**"A novel replication-restricted adenovirus for the treatment of breast cancer that allow controlled replication of E1a-deleted adenoviral vectors"**) that is attached. A summary of the work completed for this task follows.*

The initial experiments demonstrate that the bcl-xs adenovirus selectively kills cancer cells, while sparing many non-cancerous cells. One normal cell type that is killed by this virus are replicating endothelial cells. Such cells are critical for formation of the vascular system of cancer cells. In the final year of the grant, we have developed a novel delivery system for the bcl-xs adenovirus.

RESULTS

Construction and characterization of a promoter that responds to estrogens and hypoxia.

To obtain a promoter that would be preferentially active in breast cancer cells, we used a construct containing a fragment of the human pS2 promoter (-90/+10), plus two EREs that confer the estrogen responsiveness to the promoter subcloned upstream this fragment (Berry et al., 1989). The pS2 protein is a member of the trefoil factors which are normally expressed in the mucosa of the gastrointestinal tract (Ribieras et al., 1998). In addition, approximately 50% of human breast cancers show ectopic expression of pS2/TFF1 (Rio and Chambon, 1990), generally in Estrogen Receptor-positive (ER+) cases. In fact, pS2 levels increase after estrogen treatment in the human breast cancer cell line MCF7 (Jeltsch et al., 1987). The combination of the pS2 5'-flanking sequence and the EREs ensures a strong activation of the promoter in response to estrogens, only in ER+ cells (Ekena et al., 1998). Apart from this tissue-specificity, we included several HREs to enhance the activity of the promoter in the hypoxic environment of tumors. We previously determined that the addition of three HREs to an herpes simplex virus thymidine kinase promoter was sufficient to achieve hypoxic responsiveness in different cell lines (data not shown). Therefore, we introduced a cassette containing three HREs upstream of the two EREs, using a ClaI site as described in Materials and Methods. We subcloned the hole construct into a luciferase reporter plasmid (pGL2-Basic), and analyzed the activation by 17β-estradiol. To determine the response to hypoxia, we treated the cells with $CoCl_2$. Cobalt is able to trigger the hypoxic response in vitro, probably by interaction with the hem group of the proteins that act as hypoxic sensors in

the cells (Bunn and Poyton, 1996). The figure 1A shows the structure of the plasmid containing the luciferase gene under the control of the estrogen/hypoxia responsive promoter (pBERE/HRE). Two different ER+ human breast cancer cell lines (MCF7 and T47D) were transfected with the pBERE/HRE plasmid in a culture medium depleted of estrogens, and some wells were treated with 17β-estradiol and $CoCl_2$ as indicated in the figures 1B and 1C. Both cell lines responded to estrogens and hypoxia, and both stimuli showed an additive effect which reached avobe 100 fold induction over basal luciferase activity. When the cells were treated with the antiestrogen 4-0H-Tamoxifen, the estrogenic stimulation was abolished. On the other hand, the ER- breast cancer cell line was able to respond to $CoCl_2$, but not to estrogen treatment (figure 1D).

Construction of Ad5HyERE, an adenovirus with E1a and E4 regions controlled by the ERE/HRE promoter.

The data presented in figure 1 indicate that the ERE/HRE promoter is suitable for directing the expression of a gene to ER+ cells and other cells growing in hypoxic environments. In order to obtain an adenovirus that will replicate preferentially in these circumstances, we placed the E1a and E4 regions of the virus under the control of this promoter. The wild type promoters and enhancers were deleted using a PCR strategy as described in Materials and Methods. The deletions comprise nucleotides 35575 to 35818 in the E4 promoter, and nucleotides 341 to 499 in the E1a promoter. The packaging-enhancer region of the adenovirus is located between the left inverted terminal repeat (ITR) and the E1a promoter. In this region, the sequences necessary for efficient packaging of the viral genome overlap with an E1a enhancer. The enhancer consists of an element (called enhancer element II) flanked by two elements I. Enhancer element I specifically stimulates E1a transcription because it can be recognized by the nuclear factor EF-1a. The element II enhances the transcription of all the early units (Hearing and Shenk, 1986). The packaging signal is composed by repeated elements that are functionally redundant (Grable and Hearing, 1990). Since the consensus for both the enhancer and packaging functions have been already defined (Schmid and Hearing, 1997), we were able to introduce mutations that affected the enhanced elements, while preserving the packaging signals. Figure 2A shows a squematic representation of the modifications introduced in the sequence of the adenovirus type 5. The plasmid was digested with PacI in order to liberate the viral DNA, and it was transfected into 27-18 packaging cells that complement the putative lack of E1a and E4 expression. (Gao et al). When the cells showed a cytophatic effect (CPE), suggesting productive viral infection, they were collected and the viral particles were libertated after three rounds of freezing and thawing, as described in Materials and Methods. Individual plaques were then isolated using this lysate, and the virus was amplified and purified using a CsCl gradient. The viral DNA was isolated and the modifications were confirmed by PCR. (figure 2). In the E1a region, the wild type adenoviral DNA yields a fragment of 500 bp, whereas the Ad5HyERE fragment is 640 bp because the deletion of the E1a region comprises 158 bp. In the case of the E4 region, the difference in size between both viruses is not as marked (700 bp for wild type and 750 for Ad5HyERE). To further verify the presence of the ERE/HRE promoter and eliminate the possibility of contamination with wild type virus, the fragments were isolated and digested with ClaI. This is a restriction enzyme whose recognition sequence is absent in the wild type promoters, but exists in the ERE/HRE

promoter. As shown in figure 2, ClaI excised the PCR fragments of Ad5HyERE, but not the wild type adenovirus.

The function of a promoter can be drastically affected by the presence of other regulatory elements, especially in the case of a complex viral genome (Shi et al., 1997; Vassaux et al., 1999). Therefore, verified if the ERE/HRE promoter is able to regulate the expression of the E1a and E4 units in the context of the adenoviral genome. To do this, we infected monolayers of MCF7 cells with Ad5HyERE at a MOI of 5 virus/cell in the absence or presence of estrogens and $CoCl_2$. Nine hours after infection, total RNA was extracted and the transcriptional activation of E1a and E4 regions was analyzed by Northen blot using specific probes. There is a strong activation of the E1a and E4 mRNAs after estrogen treatment in this breast cancer cell line, which is drastically inhibited by the concomitant addition of the antiestrogen 4OH-Tamoxifen (figure 3). Although less efficient, chemically-induced hypoxia signal showed the same effect. To verify the data obtained with $CoCl_2$,treatment, the cells were exposed to hypoxic conditions (1% O_2 and 6% CO_2), 12 hours before and 9 hours after infection. Norhern blot analysis was performed as indicated above. The result shown in figure 3B confirms the increase of E1a mRNA transcription observed with $CoCl_2$ treatment. These data indicate that the E1a and E4 transcription units can be activated by an exogenous promoter after the deletion of the wild type activator. In addition, the ERE/HRE promoted retained its ability to respond to estrogens and hypoxia.

The cytophatic effect of Ad5HyERE on
different cell lines correlates with the expression of the Estrogen Receptors.

To analyze if the modifications introduced in the virus affected its capacity to cause CPE and death of the cells in correlation with their ER status, we compared the effect of Ad5WT and Ad5HyERE in four representative human cancer cell lines. Three of them (MCF7, T47D and MDAMB231) are derived from breast cancer, whereas HeLa is an ER- cell line derived from a cervical cancer which shows high permissivity for infection with the wild type adenovirus. The cells were infected at a multiplicity of infection (MOI) of 5 virus/cell, in the presence of 17βestradiol in the culture medium. Cells were photographed 4 days after infection, and microphotographies of representative 100X magnification fields are shown in figure 4. After infection wirth Ad5WT, all cell lines presented evident CPE with disruption of the monolayer, rounding and dettaching of cells. There are differences among the cell lines, with MCF7 being more resistant than MDAMB231, T47D or HeLa. However, Ad5HyERE was able to cause CPE in this cell line, as well as in the other ER+ cells, T47D. In contrast, when the cells don't express ER, Ad5HyERE is attenuated, as can be seen in the monolayers of MDAMB231 and HeLa cells. This demonstrates that the mutations introduced in the packaging-enhancer region are compatible with an efficient viral production, and that the regulation of the E1a and E4 transcription units has the desired effects on the behaviour of the virus. In order to better quantify this effect, we infected the same panel of cells and analyzed their viability using the MTT assay. Using this colorimetric assay, the absorbance at 600 nm is proportional to the number of viable cells, and it allows the comparison of the killing capacity of the virus on different cell lines. The cells were infected with Ad5WT or Ad5HyERE at a MOI of 5 virus/cell in the presence of 17βestradiol. The viability of the cells was measured 3 days after infection (figure 5A). In agreement with the CPE assay described above, Ad5HyERE reduced the viability of MCF7 and T47D cells as efficiently

as Ad5WT ($p>0.05$). On the other hand, MDAMB231 and HeLa cells were at least two times more resistant to the cytotoxic effect of Ad5HyERE compared to the wild type virus. The result of this assay indicates that Ad5HyERE is fully active in ER+ cells, whereas there is a significant ($p<0.001$) attenuation of the cytotoxic effect in the ER- cells. When the same assay was performed at longer times postinfection, a higher proportion of ER+ cells could be eliminated, and still the selectivity of the effect was mantained (data not shown).

Use of Ad5HyERE to allow replication of an E1-deleted adenovirus.

Apart from being a therapeutic tool itself, Ad5HyERE can be used to complement the E1a defficiency of already existing replication-incompetent adenoviral vectors. There are several E1a-deleted adenovirus engineered to deliver lethal genes to cancer cells. Although they can efficiently deliver the gene and obtain high levels of expression that lead to the death of the infected cells, the cells that escape the initial infection are not affected and cause the relapse of the tumor. This problem is especially important if the readministration of the virus can be compromised by the development of antibodies, as is the case in humans. The expression of E1a proteins in Ad5HyERE is controlled by a promoter that is preferentially activated in ER+ cells and others growing under hypoxic conditions. It means that if a cell is coinfected with Ad5HyERE and an E1a-deleted adenovirus, the same regulation of replication can apply to both viruses because Ad5HyERE will provide the E1a protein in trans. This hypothesis, has been recently demonstrated using an E1b-deleted replication-competent adenovirus (Motoi et al, 2000). To verify this possibility in our system, we used an E1a-deleted adenovirus vector carrying the β-galactosidase gene under the control of a constitutive RSV promoter (Ad5LacZ), which allowed a quantitation of the complementation. T47D cells were infected simultaneously with a suboptimal amount of Ad5LacZ (MOI=20), and Ad5HyERE (MOI=0.5) in the presence of 17βestradiol or $CoCl_2$ as indicated in the legend for figure 6. Two days later, the monolayers were fixed and incubated with the substrate X-Gal to identify the cells that were infected with Ad5LacZ. In the microphotographies of figure 6A, the positive cells are darker than the uninfected ones because of the blue color of the modified X-Gal substrate. The average number of blue cells per field were counted in duplicate wells and the results are graphically reprsented in figure 6B. The addition of Ad5HyERE stimulated more than 3 times the number of cells infected by Ad5LacZ in the first 48 hours. If the cells are incubated for longer periods, this effect is amplified and virtually 100% of the cells are infected with the same initial amount of Ad5LacZ (data not shown). The cooperation is mediated by the regulated expression of the E1a protein under the control of the ERE/HRE promoter, because the antiestrogen 4-0H-Tamoxifen inhibits the complementation, and the treatment with $CoCl_2$ stimulates it (figure 6).

The above results demostrate that a controlled replication of an E1-deleted adenovirus can be obtained by suplementation of the E1a protein with a replication-conditioned adenovirus. Next, we determined whether this strategy could be used to amplify the killing capacity of an E1a-deleted adenovirus vector that expresses the pro-apoptotic gene Bcl-xs (Ad5Bcl-xs). It has been previously demostrated that this virus can induce apoptosis in MCF7 cells in culture, and it inhibits the growth of tumors induced by injection of these cells in nude mice (Clarke et al., 1995; Ealovenga et al., 1996). However, multiple administrations of the virus were necessary to keep the inhibitory effect. The addition of a replication-conditional adenovirus such as Ad5HyERE could

enhance the cytotoxic effect of Ad5Bcl-xs an allow the use of lower doses of this vector. To check this possibility, we infected MCF7 cells with Ad5Bcl-xs at MOI of 2 and 20 virus/cell, which is an amount of virus 5000-500 times lower than the dose needed to effectivelly kill these cells in culture. Additional monolayers were infected with Ad5Bcl-xs plus Ad5HyERE at a MOI of 0.5 virus/cell in the presence of 17βestradiol, with or without 4-0H-Tamoxifen. It has to be noted that neither Ad5Bcl-xs nor Ad5HyERE used separately show significant cytotoxicity when used at these low titers. The cells were re-fed every three days, and the effect was analyzed 8 days after infection. As shown in figure 7A, Ad5HyERE enhanced the killing effect of Ad5Bcl-xs at MOI as low as 0.5 virus/cell. Again, the complementation elicited by Ad5HyERE can be specifically inhibited with the antiestrogen 4-0H-Tamoxifen. In the figure 7B, the cells were treated basically as described above, but their viability was analyzed after 8 days of culture using the MTT assay. Ad5Bcl-xs was used at a higher MOI (200 virus/cell), and the MOI of Ad5HyERE was as low as 0.25. The result indicates that this concentration of Ad5Bcl-xs was not lethal for MCF7 cells, whereas Ad5HyERE caused slight ($p=0.07$) decrease of viability. However, when both viruses were combined, 90% of the cells died at this time point. The addition of 4OH-Tamoxifen caused a significant ($p<0.005$) protection from the lethal effect of this combination of viruses. In addition, a control E1a-deleted adenovirus without exogenous gene expression was coinfected with Ad5HyERE in the same conditions. In contrast with Ad5Bcl-xs, no significant ($p=0.08$) decrease in the viability of the cells was observed with this combination. This indicates that the amplification of the killing efect of Ad5Bcl-xs depends on the expression of the proapoptotic gene, and is not only the consequence of the replication of the vector. These results suggests that the effect of E1a-deleted adenoviral vectors can be amplified in a controlled fashion by using a replication-conditional adenovirus that suplements the E1a protein

In vivo amplification of the bcl-xs adenovirus. The ability of Ad5HyERE and Bcl-xs to kill tumors *in vivo* was tested. MCF-7 and T47D tumors were established in nude mice. These tumors were injected with Ad5HyERE alone, Ad5HyERE and either AdBcl-xs or a control replication deficient adenovirus, or AdBcl-xs alone. The combination of Ad5HyERE and Bcl-xs resulted in significantly greater shrinkage of the tumors compared to any of the other groups (data not shown).

FIGURE LEGENDS

FIG. 1. Construction and characterization of the estrogen and hypoxia-inducible promoter (ERE/HRE). A. Squematic representation of pBERE/HRE, a reporter plasmid expressing the luciferase gene under the control of the ERE/HRE promoter. The ERE and HRE consensus are indicated. The arrow represents the pS2 promoter fragment (nucleotides -90/+10). Two EREs are subcloned upstream this region (dark dotted box), followed by three HREs introduced in a ClaI site (white dotted box). B, ER+ T47D human breast cancer cells were transfected with 0.5μg pBEREHRE plasmid together with 0.3μg of a βgalactosidase reporter plasmid to correct for efficiency of transfection. Phenol Red-free medium and 5% charcoal-dextran stripped serum were used. After 12 hours, the medium was changed and cells were left untreated, or treated with 2.5 nM 17βestradiol(E),100 μM $CoCl_2$ (Co), or 1 μM 4OH-Tamoxifen (T), as indicated in the horizontal axis of the graphic. Twelve hours later, cells were lysed and luciferase activity

was measured in protein extracts together with βgalactosidase activity. The bars represent the Relative Luciferase Units (RLU), divided by the absorbance at 410 nm, as described in Materials and Methods. Panels C and D show the results of same assay performed in the ER+ MCF7 and the ER- MDAMB23 human breast cancer cell lines, respectively. The assay was repeated three times with similar results.

FIG. 2. Construction of Ad5HyERE. A. Squematic representation of the modifications introduced in the adenovirus type 5 genome. The size of the viral regions are not proportional in the squeme. In the left end of the genome, the E1a enhancer/packaging signal is represented as a black box. Its sequence is displayed below. The E1a enhancer elements are distinguised by capital letters, and the changes in nucleotides are indicated above. The packaging elements are distinguised by bold characters. The deleted E1a and E4 promoters are substituted by the ERE/HRE promoter. The modified regions are reproduced below to indicate the ClaI site in the artificial promoter, and the position of the PCR primers used in order to verify the structure of the virus. B, DNA was extracted from Ad5WT and Ad5HyERE, and PCR reactions were performed using primers 1 and 1' (panel 1), or primers 2 and 2' (panel 2). The + sign indicates digestion of the PCR fragment with the restriction endonuclease ClaI. The fragments were resolved in a 1.5% agarose gel.

FIG. 3. Analysis of E1a and E4 transcription in Ad5HyERE-infected MCF7 cells. A. MCF7 cells were mantained in Phenol Red-free medium with 2.5% charcoal-dextran stripped serum, and pretreated for 12 hours with 2.5 nM 17βestradiol (E), 2.5 μM 4OH-Tamoxifen (Tam), or 150 μM $CoCl_2$ as indicated in each lane. Cell were then infected with Ad5HyERE at a MOI of 5 virus/cell and total RNA was extracted after 9 hours of infection. Northern blot analysis was performed using radioactive probes specific for the E1a, E4 regions or the constitutively expressed βactin gene as a control. B. E1a transcription activation under hypoxic conditions. MCF7 cells were placed in an hypoxia chamber (1% O_2, 6% CO_2) 12 hours prior infection, cells were then infected for 1 hour in normal conditions, and then mantained under hypoxia for 9 additional hours before the samples were collected and Northern blot was performed as in figure 3A.

FIG. 4. Cytophatic effect of Ad5HyERE on different cell lines. The ER+ cell lines MCF7 and T47D, and the ER- cell lines MDAMB231 and HeLa were infected with Ad5WT or Ad5HyERE at a MOI of 5 virus/cell in the presence of 1.5 nM 17βestradiol. The monolayers were photographed 4 days after infection. Representative 100X magnification fields are shown. The control panels are non-infected cells mantained in identical conditions. The assay was repeated four times with similar results.

FIG. 5. Viability of different cell lines infected with Ad5HyERE. A. The same panel of cells described in figure 4 were left uninfected (black bars), infected with Ad5WT (stripped bars) or with Ad5HyERE (gray bars) at a MOI of 5 virus/cell in the presence of 1.5 nM 17βestradiol. Three days after infection, the viability of the cells was analyzed using the MTT assay as described in Materials and Methods. The bars indicate the absorbance at 600 nm, which is proportional to the number of viable cells. Each bar represents the mean of four individual wells. The assay was repeated two times with similar results. B. The same assay described in figure 5A was performed on MCF7 and

HeLa cells, using a MOI of 3 virus/cell of Ad5WT or Ad5HyERE. Fresh medium with 17βestradiol was added every 3 days, and the viability of cells was measured on day 10 postinfection.

FIG. 6. Complementation of an E1a-deleted adenovirus vector with Ad5HyERE. A. T47D cell were infected with a suboptimal amount (MOI of 20 virus/cell) of a replication-defficient E1a-deleted adenoviral vector expressing the βgalactosidase gene (Ad5LacZ), panel 1 (LacZ). In panels 2 to 5, the same amount of Ad5LacZ was combined with Ad5HyERE at a MOI of 0.5 virus/cell (LacZ+HyERE). Cells were treated with 2 nM 17βestradiol (E), 17βestradiol plus 2.5 μM 4OH-Tamoxifen (E+T 2.5), 17βestradiol plus 10 μM 4OH-Tamoxifen (E+T 10), or 150 μM $CoCl_2$ (Co). Two days after infection, the monolayers were fixed and incubated with X-Gal as described in Materials and Methods. Representative 400X magnifiction fields were photographed. B, Quantification of the number of blue cells/field. The bars represent the average of five different fields. The assay was repeated three times with similar results.

FIG. 7. Use of Ad5HyERE to amplify the lethal effect of an E1a-deleted adenovirus vector expressing the proapoptotic gene Bcl-xs. A. MCF7 cells were infected with suboptimal amounts of Ad5Bcl-xs (MOI of 2 and 20 virus/cell, as indicated in the figure), in the presence of 1.5 nM 17βestradiol. In some of the monolayers, Ad5HyERE was added at a MOI of 0.5 virus/cell. When indicated, 2 μM 4OH-Tamoxifen was added. Panel 1 shows uninfected cells, and panel 2 shows cells infected with Ad5HyERE alone. The cells were re-fed with new medium every three days, and representative 400X magnification fields were photographed after 8 days of infection. The experiment was repeated three times with similar results. The significance of the differences observed was statistically analyzed using the *t* test.

CONCLUSIONS

Two major advances have resulted from this project. First, a sensitive PCR-based assay for keratin 19 has been developed and been shown to detect breast cancer cells in the bone marrow and in circulating blood. Keratin 19 appears to be a novel and effective marker for RT-PCR detection of breast cancer cells in peripheral blood and the bone marrow. The collection of patient samples now exceeds one hundred RNA preparations. This includes patients with stage I through IV breast cancer, and includes more than fifty patients that have undergone BMT. Next, the bcl-xs adenovirus has been shown to selectively kill breast cancer cells while sparing normal cells. We envision human breast cancer clinical trials to begin in the near future.

As an adjunct to surgery, radiation, or chemotherapy, autologous bone marrow transplants (BMTs) are increasingly used as a method to increase survival of patients with aggressive non-hematopoietic tumors. However, retroviral tagging and PCR studies indicate that autologous marrow is often the source of cancer relapse in these patients. Several methods have been devised to purge marrow of tumor cells prior to transplantation, but each has distinctive shortcomings. Immunologic methods depend on a unique tumor cell surface epitope and a high avidity antibody for efficient negative selection. Chemical techniques can have significant hematopoietic toxicity. We have previously shown that adenovirally mediated transient expression of *bcl-x_S*, a functional repressor of *bcl-2*, would induce PCD in contaminating tumor cells found in bone marrow cell preparations. It is reported here that a pure population of hematopoietic stem cells does not express a transgene when exposed to a recombinant adenovirus. We further postulated that hematopoietic stem cells would retain the ability to repopulate hematopoiesis following treatment with adenoviral vectors.

We show that a combination of *bcl-x_S* and TK adenovirus are the most effective and least toxic method of killing MCF-7 cells in a mixed tumor/hematopoietic *ex vivo* culture. Of all tumor cell types we have tested to date, MCF-7 cells have proven to be among the most resistant to *bcl-x_S* adenovirus treatment [25]. By combining a TK adenovirus/ganciclovir treatment with *bcl-x_S* adenovirus infection of mixed tumor/marrow cultures, MCF-7 cells are purged from the marrow to below detectable levels. In addition, the combination TK/ *bcl-x_S* treatment results in an equally efficient purging of hematopoietic progenitors compared to an equivalent treatment using only *bcl-x_S*, while resulting in a progenitor cell toxicity that is equal or slightly less. This combined treatment minimizes the non-specific hematopoietic toxicity of these adenoviral vectors, while preserving MCF-7 purging efficiency, although TK treatment alone may be a useful treatment. This data thus supports the hypothesis that *bcl-x_S* overexpression mediated by adenoviral vectors may be used to effectively purge solid tumor cells from human bone marrow. Although the mechanism behind this observation is unclear, to date MCF-7 cells have proven to be the only cell type for which a combined *bcl-x_S*/TK infection is required to completely purge human marrow *in vivo*. Other cancer cell line, such as the SHEP 1 neuroblastoma line, are efficiently purged by *bcl-x_S* viral MOIs that do not have any significant effect on hematopoietic cell activity (25).

In this work, hematopoietic stem and progenitor cells are shown to exhibit resistance to greater MOIs than that required to infect 100% of neuroblastoma cells with

an adenovirus marker gene. Previous work has shown that mouse hematopoietic stem cells can be isolated on the basis of the phenotype Thy-1.1^{lo} Lin^{-} Sca-1^{+} [30]. As few as 30 of these cells can rescue 100% of lethally irradiated mice, producing long-term, multilineage reconstitution (26). By purifying homogeneous populations of murine stem cells, we were able to directly target them *in vitro* with adenovirus at MOIs greater than those necessary to kill tumor cells. After such treatment, murine stem cells retained their viability but did not express the adenoviral LacZ gene, whereas tumor cells expressed this marker. Confirming and extending this data, our *in vivo* studies show that murine marrow infected with bcl-x_S, TK, or LacZ adenovirus retains the capacity for long term, apparently multilineage, engraftment upon transplant into lethally irradiated syngeneic mice.

The ability to selectively kill tumor cells, while sparing all of the hematopoietic cells in bone marrow prior to autologous transplantation represents a novel method in purging/transplantation as a treatment of many human neoplasms. Previous methods involving immunologic, mechanical, or chemical based tumor purging have had limited success, require extensive marrow processing, or are useful for treatment of only one specific cell type. We conclude that bone marrow purging using an adenoviral-based method (that can be used alone or in conjunction with other purging strategies) represents a simple, quick, and efficient method for purging a wide variety of non-hematopoietic tumor cells while retaining hematopoietic stem cell activity.

The bcl-x_s adenovirus will begin toxicology testing for the FDA this year. We anticipate clinical breast cancer clinical trials to begin with this virus within one to two years.

The use of tumor-specific promoters to control the expression of viral genes is a promsing method for the construction of therapeutic viruses. The lack of well characterized promoters limits the systematic aplication of this technique. We are interested in the development of a replication-conditional adenovirus for the treatment of breast cancer. In this study our goal was to explore the feasibiliy of using an arificial promoter combining individual elements that provide tissue and tumor specificity. As an initial approach to direct the replication of the virus to breast epithelial cells, we used a promoter containing EREs. Only in cells that express ER this promoter can be activated by the ciruculating estrogens. Although the expression of these receptors is not exclusive tobreast tissue, they are useful to conceptually validate the strategy until more specific elements are identified.The use of EREs may also offer several advantages. First, this strategy allows the exogenous modulation of the response by administration of antiestrogens such as Tamoxifen, which is a well tolerated drug extensively used in humans for the treatment and prevention of hormone-sensitive breast cancers. In addition, if a local or regional treatment is envisioned, the ERs can provide a preferential replication of the virus in the breast epithelial cells versus the surrounding parenchima. Finally, although many other tissues can express ER, it is not clear that in all the cases the levels and subtypes of receptors (ERα or ERβ) can stimulate the viral replication.

In the Ad5HyERE virus, two early transcription units (E1a and E4) are conditionally expressed. As both of them are necessary for an efficient viral

replication,we believe that this contributes to the control of the virus and constitutes a safety mechanism to prevent the appearance of wild type revertants. Moreover, the E4 functions have been implicated in the virus-induced CPE and death of infected cells. In this way, the control of the E4 orfs expression can direct not only the replication, but also the lethal effect of the virus to the cancer cells, which is the final goal of the therapeutic viruses. Compared to other replication-restricted adenoviruses, another new feature of Ad5HyERE is the inactivation of the endogenous E1a enhancer. We have demonstrated that this is compatible with an efficient viral production because the packaging sequence was preserved. This modification may eliminate the non-regulated influence of the upsteam elements on the E1a transcription, and increase the therapeutic index of the virus.

The HREs were included in the promoter to facilitate the replication of the virus in the hypoxic environment of the tumors. This is one of the few characteristics that is shared by virtually all solid tumors. We hypotesize that this elements can be used alone in the context of a minimal promoter, or combined with other recognition sequences. The data shown with the ERE/HRE promoter demonstrate that both elements can independently stimulate the expression of genes in response to their particular activators, and achieve an additive effect when both are present. This method can provide great versatility to the development of new targeted expression systems for cancer gene therapy.

Replication-conditional adenoviruses can act as a therapeutic tool themselves, but they can also deliver lethal genes to cancer cells to enhance their effect. In this way, the addition of a cassette for the expression of proapoptotic genes or prodrug-converting enzymes like tymidine kinase or cytosine deaminase is technically affordable (Wildner et al., 1999). However, another possibility is the use of a single replication-conditional adenovirus to amplify the effecto of any E1a-deleted adenoviral vector (Motoi et al., 2000). We confirmed this hypothesis using the Ad5hyERE in combination with a replication-defficient vector carrying the βgalactosidase gene. Finally, we used the same strategy to enhance the cytotoxic effect of a virus expressing the proapoptotic gene Bcl-xs.

In summary, the combination of tissue and tumor-specific elements is a promising method for the construction of replication-conditional viruses that can revitalize several cancer gene therapy strategies.

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PERSONNEL RECEIVING PAY FROM THIS EFFORT
DAMD17-95-1-5021
(January, 1998 through January, 1999)

Paul Adams	01-01-98 through 12-31-98
Michael F. Clarke, M.D.	01-01-98 through 01-31-99
Jennifer Sanderson	01-01-98 through 10-31-98
Shun-Hsin Liang	01-01-98 through 01-31-99
Preston Ford	01-01-98 through 09-30-98

Figure 1

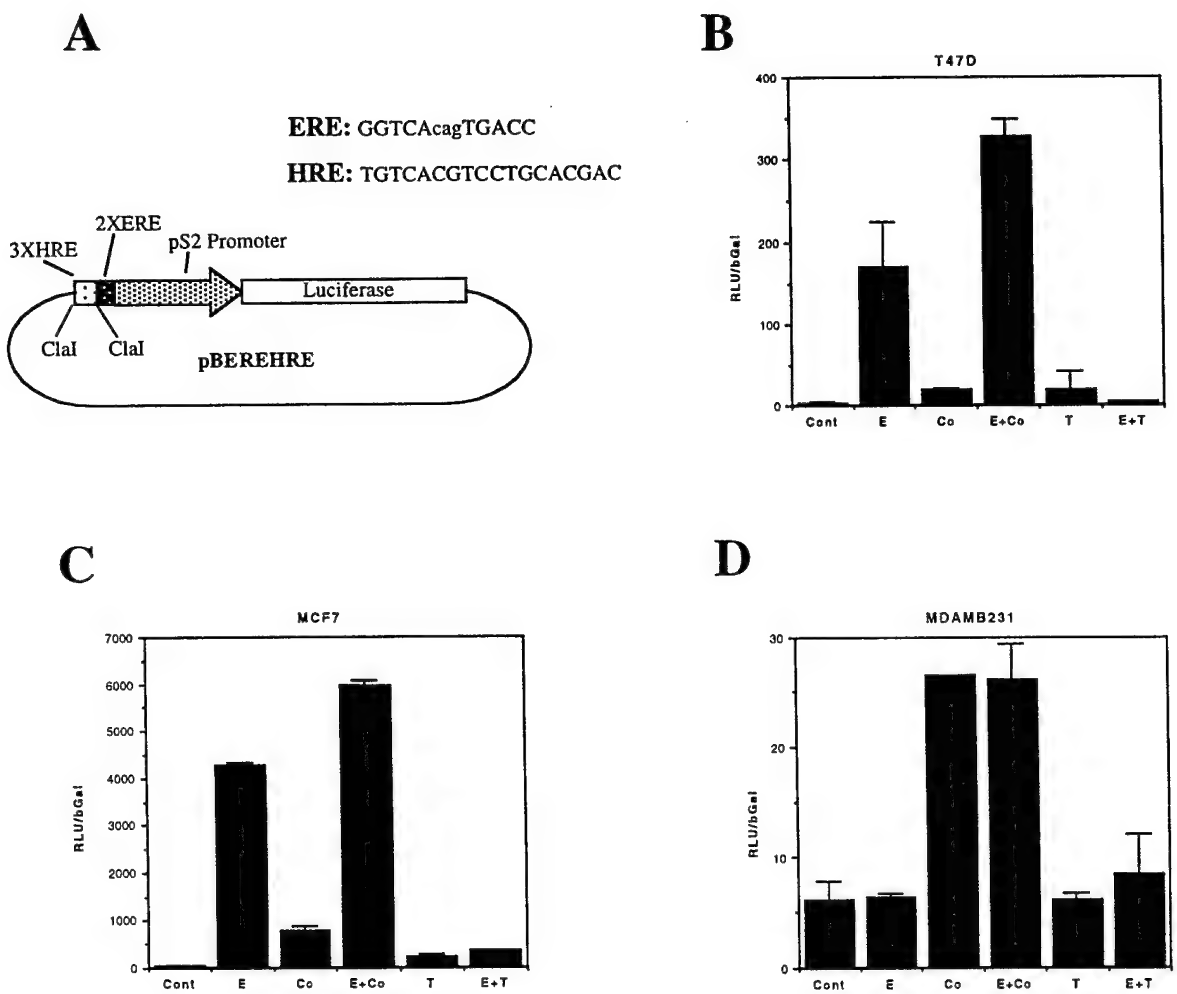

A
ERE: GGTCAcagTGACC
HRE: TGTCACGTCCTGCACGAC
2XERE
3XHRE
pS2 Promoter
Luciferase
ClaI
ClaI
pBEREHRE
B
T47D
RLU/bGal
Cont
E
Co
E+Co
T
E+T
C
MCF7
RLU/bGal
D
MDAMB231
RLU/bGal

Figure 2

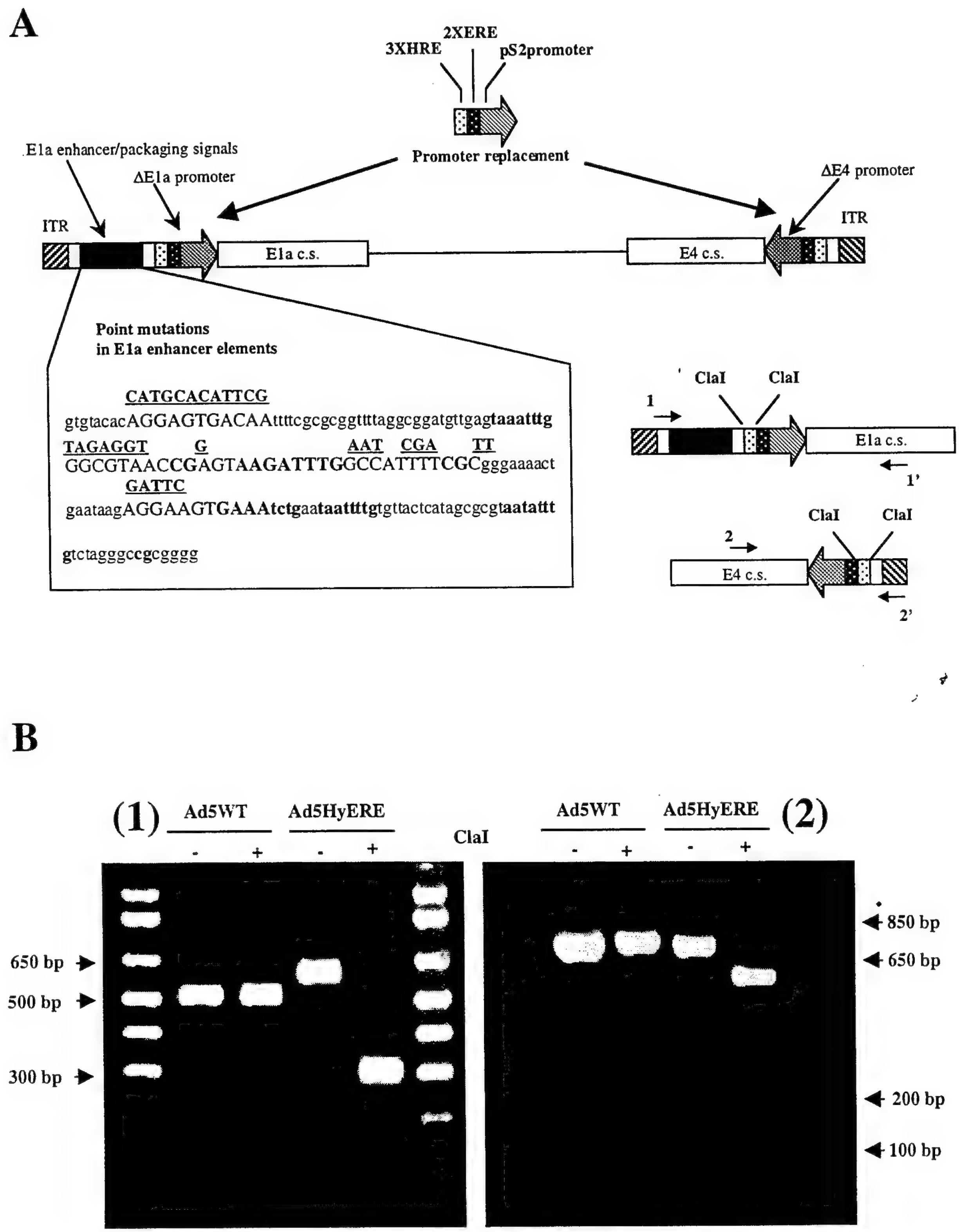
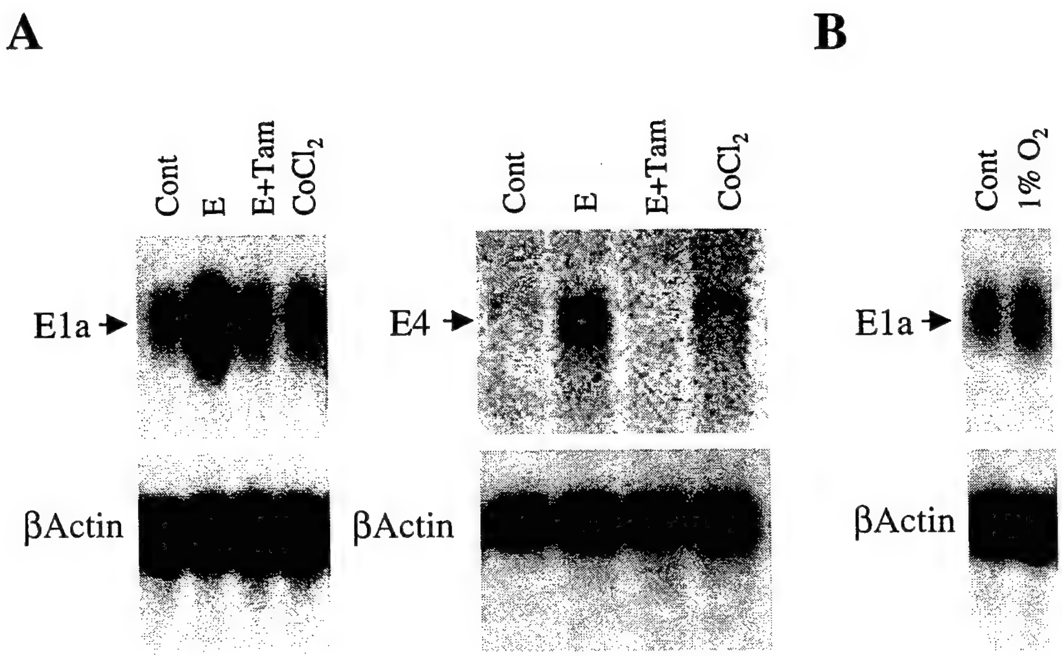

A
2XERE
3XHRE
pS2promoter
E1a enhancer/packaging signals
ΔE1a promoter
Promoter replacement
ΔE4 promoter
ITR
E1a c.s.
E4 c.s.
ITR
Point mutations
in E1a enhancer elements
CATGCACATTCG
gtgtacacAGGAGTGACAAttttcgcgcggttttaggcggatgttgagtaaatttg
TAGAGGT G AAT CGA TT
GGCGTAACCGAGTAAGATTTGGCCATTTTCGCgggaaaact
GATTC
gaataagAGGAAGTGAAAtctgaataattttgtgttactcatagcgcgtaatattt
gtctagggccgcgggg
ClaI
ClaI
1
E1a c.s.
1'
ClaI
ClaI
2
E4 c.s.
2'
B
(1)
Ad5WT
Ad5HyERE
ClaI
- + - +
650 bp
500 bp
300 bp
Ad5WT
Ad5HyERE
(2)
- + - +
850 bp
650 bp
200 bp
100 bp

Figure 3



A
B
Cont
E
E+Tam
CoCl2
E1a
βActin
Cont
E
E+Tam
CoCl2
E4
βActin
Cont
1% O2
E1a
βActin

Figure 4

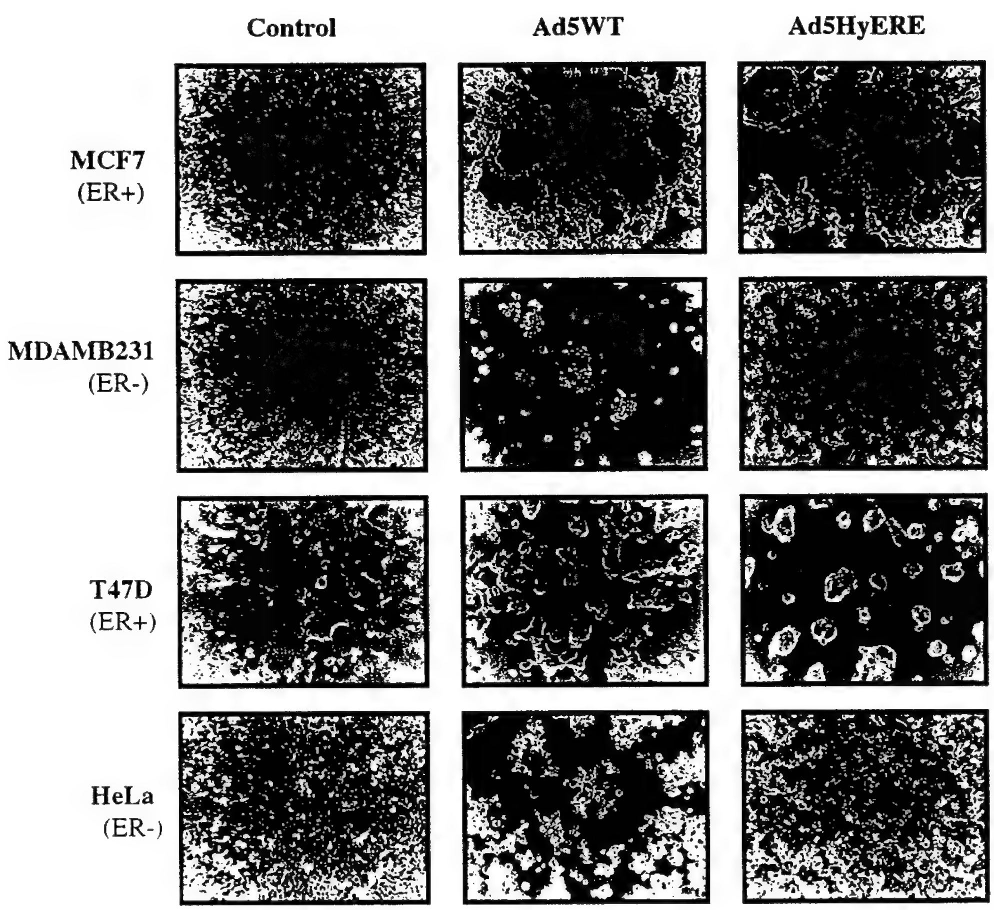


Figure 5

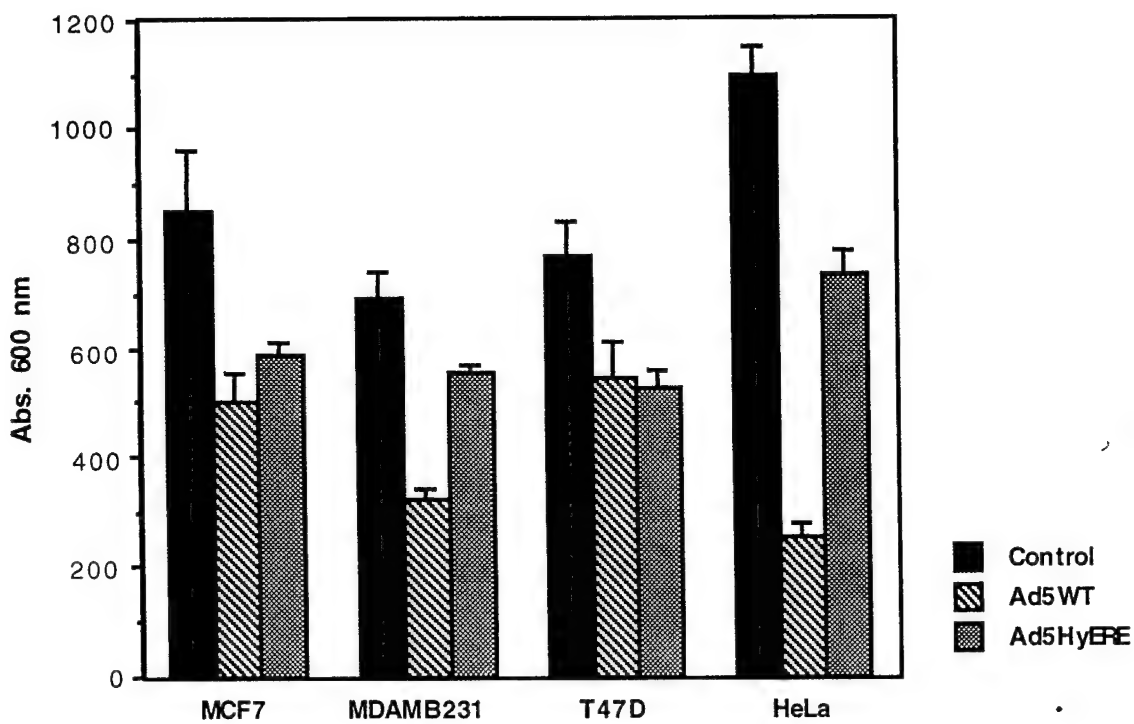
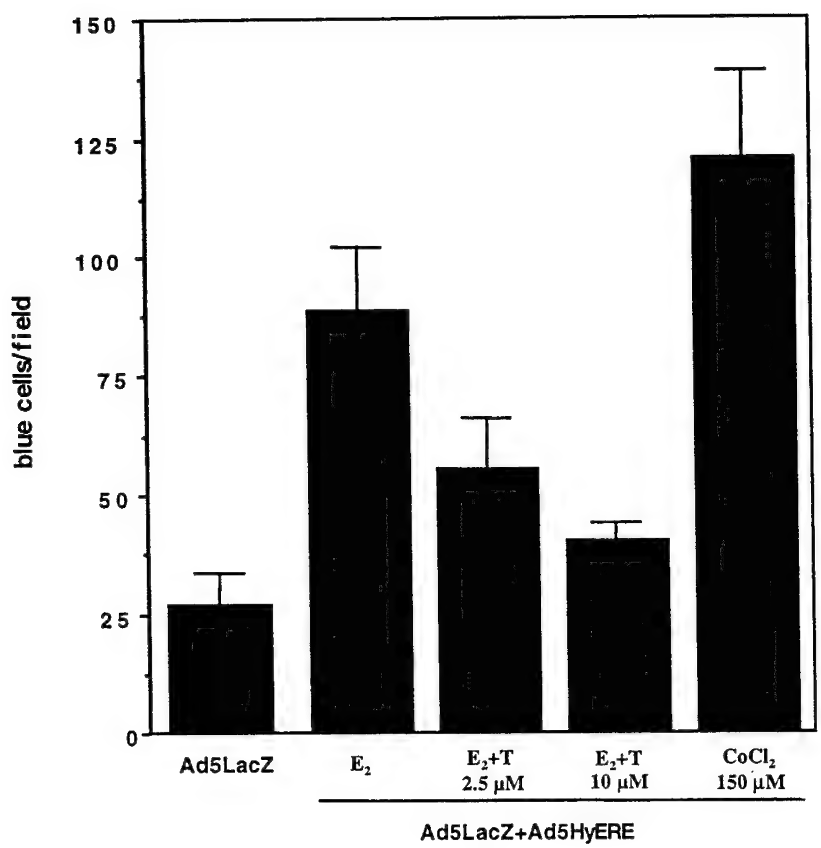

1200
1000
800
600
400
200
0
Abs. 600 nm
MCF7
MDAMB231
T47D
HeLa
Control
Ad5WT
Ad5HyERE

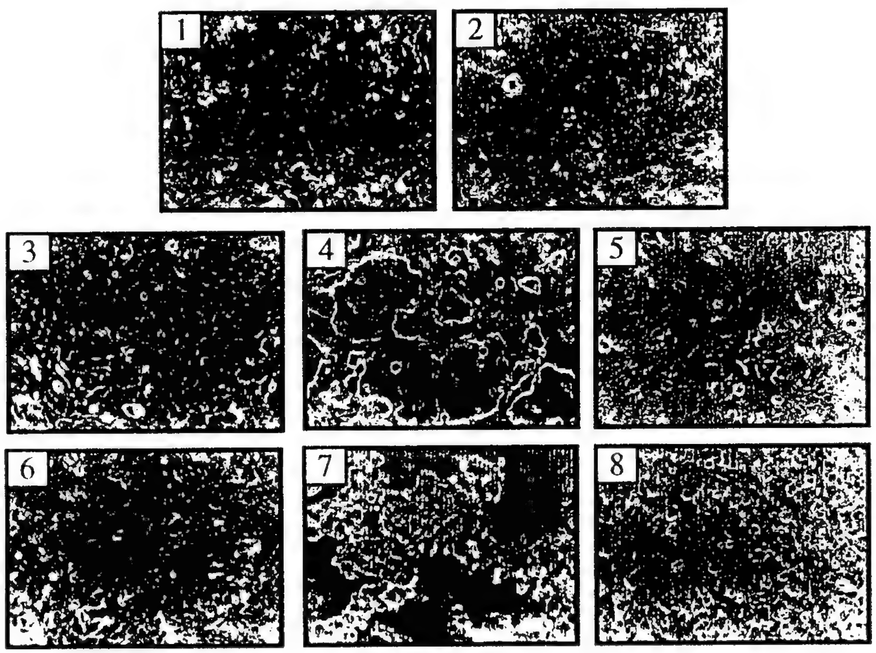
Figure 6



150
125
100
75
50
25
0
blue cells/field
Ad5LacZ
E_2
E_2+T
2.5 µM
E_2+T
10 µM
$CoCl_2$
150 µM
Ad5LacZ+Ad5HyERE

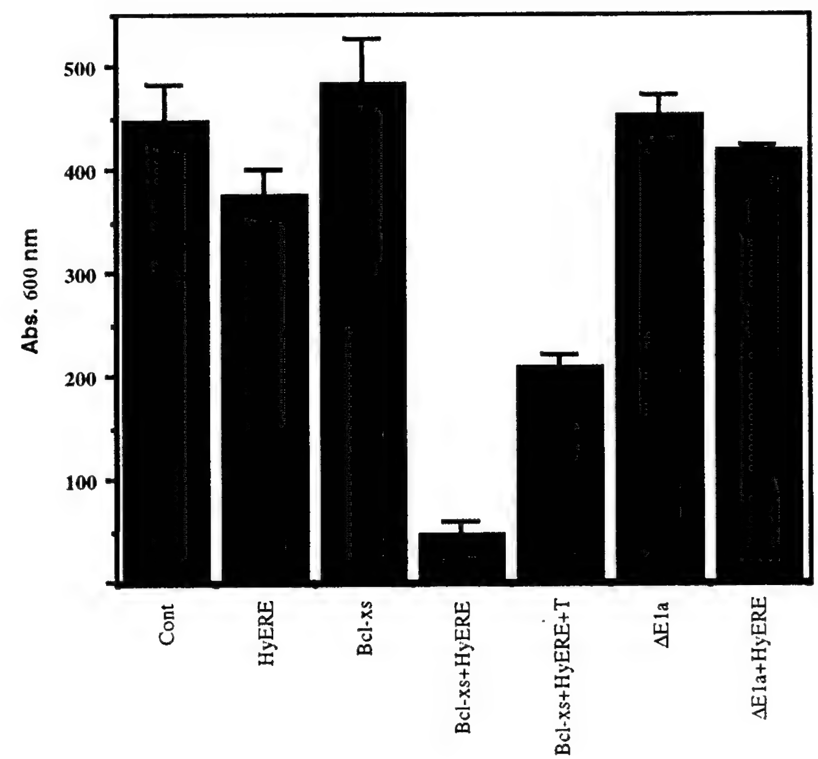
Figure 7

A



1.- Control
2.- HyERE
3.- Bcl-xs MOI 2
4.- Bcl-xs MOI 2 + HyERE
5.- Bcl-xs MOI 2 + Ad5HyERE + T
6.- Bcl-xs MOI 20
7.- Bcl-xs MOI 20 + Ad5HyERE
8.- Bcl-xs MOI 20 + Ad5HyERE + T

B



MANUSCRIPT #1

HUMAN GENE THERAPY 11:2009–2024 (September 20, 2000)
Mary Ann Liebert, Inc.

A Novel, Conditionally Replicative Adenovirus for the Treatment of Breast Cancer That Allows Controlled Replication of E1a-Deleted Adenoviral Vectors

RUBEN HERNANDEZ-ALCOCEBA, MICHAEL PIHALJA, MAX S. WICHA, and MICHAEL F. CLARKE

ABSTRACT

The efficiency of gene therapy strategies against cancer is limited by the poor distribution of the vectors in the malignant tissues. To solve this problem, a new generation of tumor-specific, conditionally replicative adenoviruses is being developed. To direct the replication of the virus to breast cancer, we have considered one characteristic present in a great proportion of these cancers, which is the expression of estrogen receptors (ERs). On the basis of the wild-type adenovirus type 5, we have constructed a conditionally replicative adenovirus (Ad5ERE2) in which the E1a and E4 promoters have been replaced by a portion of the pS2 promoter containing two estrogen-responsive elements (EREs). This promoter induces transcriptional activation of the E1a and E4 units in response to estrogens in cells that express the ERs. Ad5ERE2 is able to kill ER$^+$ human breast cancer cell lines as efficiently as the wild-type virus, but has decreased capacity to affect ER$^-$ cells. By complementation of the E1a protein in *trans*, Ad5ERE2 allows restricted replication of a conventional E1a-deleted adenoviral vector. When a virus expressing the proapoptotic gene Bc1-xs (Clarke *et al.*, Proc. Natl. Acad. Sci. U.S.A. 1995;92:11024–11028) is used in combination with Ad5ERE2, the ability of both viruses to induce cell death is dramatically increased, and the effect can be modulated by addition of the antiestrogen tamoxifen.

OVERVIEW SUMMARY

To obtain controlled replication of adenoviral vectors, the E1a transcription unit of the virus is usually placed under the control of a tumor-specific promoter. In the absence of a well-characterized tumor-specific promoter for breast cancer, we have used a portion of the pS2 promoter containing two estrogen response elements to control the expression of the E1a and E4 viral proteins. The result is a new virus (Ad5ERE2) that replicates preferentially in human breast cancer cell lines expressing estrogen receptors. Ad5ERE2 can be used to induce replication of an E1a-deleted adenoviral vector by complementation of the E1a protein in *trans*. This cooperation can be inhibited by antiestrogenic drugs such as tamoxifen.

INTRODUCTION

Adenoviruses have great potential as vectors for cancer gene therapy, especially in those strategies that rely on the transduction of malignant cells with lethal genes. In this scenario, some of the weaknesses of these vectors for use in treating inherited diseases are irrelevant. For example, the lack of a sustained expression of the therapeutic gene is not an issue because the goal is to kill the cells. Moreover, a local stimulation of the immune system may help to eradicate the malignancy. The main impediment in using adenovirus vectors for the treatment of solid tumors is the poor distribution of the vector throughout the tumor. The killing of malignant cells by targeted delivery of lethal genes has been conceptually demonstrated in many preclinical studies using different approaches (Roth and Cristiano, 1997; Dranoff, 1998). However, to achieve a clinically significant benefit from cytotoxic viruses, some sort of amplification of the initial effect is required (Han *et al.*, 1998).

In general, adenoviruses that contain suicide genes are replication incompetent by deletion of viral genes that are necessary for the replication of the virus. Adenovirus has five transcription units that are expressed before the onset of viral replication, called early units (E1a, E1b, E2, E3, and E4) (Shenk, 1996). The E1a region encodes the first two polypeptides that accumulate after viral infection. Apart from inducing the entry of the host cell into the S phase of the cell cycle, they have the ability to activate other transcription units in the viral genome

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(Lillie and Green, 1989). Without the E1a gene the virus is virtually unable to replicate, and this is usually deleted in adenovirus-derived vectors. The E4 region comprises at least seven open reading frames (ORFs) whose functions are still not completely elucidated. The consequence of the deletion of this region is an inefficient production of viral particles due to defects in viral replication, late gene expression, host cell shutoff, and particle assembly (Halbert *et al.*, 1985; Falgout and Ketner, 1987; Lusky *et al.*, 1998). Interestingly, it has been shown that the E4 ORF 4 can induce p53-independent apoptosis (Marcellus *et al.*, 1998; Shtrichman and Kleinberger, 1998).

A new generation of conditionally replicative adenoviruses, also called tumor-specific replication-restricted adenoviruses (TRRAs), has been developed to kill malignant cells in a controlled fashion (Vile *et al.*, 2000). The goal is to obtain viruses that can replicate only in the tumor. In this way, an initial dose of the therapeutic virus will infect a limited number of cells, but the natural cycle of the virus will continue in cancer cells, with the subsequent replication, death of the host cell, and liberation of thousands of new viruses. Ideally, the process goes on until no more cancer cells are available for the virus to replicate, and it limits the spread of the virus to the tumor margins. At least four different conditionally replicative adenoviruses have already been developed, demonstrating the feasibility and great potential of this new approach. To direct the replication of the virus to cancer cells, two different basic strategies have been used so far. The first one led to the construction of dl1520, an adenovirus that has a deletion in the E1b region (Bischoff *et al.*, 1996). The E1b 55-kDa protein binds and inactivates p53 in the host cell, and this is necessary for its entry in the S phase of the cell cycle. Therefore, this virus can replicate only in cells with an abolished p53 function. Although this mechanism of action is controversial (Hall *et al.*, 1998; Rothmann *et al.*, 1998; Hay *et al.*, 1999), the therapeutic responses obtained with this virus, now called ONYX-015, are promising (Heise *et al.*, 1997, 1999; Kirn *et al.*, 1998). A mutant adenovirus with a partial deletion in the E1a region has been described (Fueyo *et al.*, 2000). The deletion unables the binding to the Rb protein, and hence limits the replication of the virus to cells with a disrupted Rb pathway, which is a common characteristic of many cancer cells.

The second strategy for producing replication-restricted adenoviruses consists of placing the E1 region under the control of tissue or tumor-specific promoters. The prostate-specific promoter and enhancer have been used to direct the viral replication to prostate cancer cells (Yu *et al.*, 1999), and the α-fetoprotein gene promoter has been used to target hepatocellular carcinoma cells (Hallenbeck *et al.*, 1999). This method opens exciting possibilities for the treatment of many different malignancies, once a promoter is identified that is preferentially activated in the tumor. The search of such promoters can be optimized by using new techniques such as differential display (Peng and Vile, 1999) and serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), but the list of tumor-specific promoters is limited. An alternative approach is the use of tissue-specific promoters to restrict the replication of the virus to the affected organ. For example, in up to 70% of breast cancers, the malignant cells retain the expression of estrogen receptors (ERs) (Valavaara, 1997). In the presence of estrogens, these nuclear receptors can bind to a specific sequence (estrogen response element, ERE) present in the promoter of certain genes, and activate their transcription (Tsai and O'Malley, 1994).

In this study we describe the construction and characterization of a new conditionally replicative adenovirus for the treatment of breast cancer. We have replaced the E1a and E4 promoters of the wild-type adenovirus type 5 by a portion of the pS2 promoter, which contains two EREs. This promoter can be activated by estrogens in cells expressing ERs, and transcription can be inhibited by antiestrogens such as tamoxifen. We show evidence that this virus preferentially kills ER^+ cells and can be used in combination with an E1a-deleted adenoviral vector to amplify the delivery of a proapoptotic gene to breast cancer cells.

MATERIALS AND METHODS

Cell lines

MCF7 (ATTC HTB 22), T47D (ATTC HTB 133), and BT474 (ATTC HTB 20) are ER^+ human breast cancer cell lines. MDAMB231 (ATTC HTB 26) and SKBR3 (ATTC HTB 30) are ER^- human breast cancer cell lines. Cells were maintained in RPMI medium (BioWhittaker, Walkersville, MN) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). For MCF7, T47D, BT474, and MDAMB231, insulin (10 μg/ml; GIBCO-BRL, Grand Island, NY) was added. HeLa (ATTC CCL 2) is an ER^- human cervical cancer cell line, and it was maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% fetal bovine serum. SUM-309PE cells are ER^+ human breast cancer primary cells, and were maintained in Ham's F-12 (BioWhittaker) supplemented with insulin (5 μg/ml), hydrocortisone (1 μg/ml), epidermal growth factor (EGF, 10 ng/ml), and cholera toxin (100 ng/ml). When the experiment required depletion of estrogens, improved minimal essential medium (IMEM) without phenol red was used (GIBCO-BRL), supplemented with 2.5% charcoal-dextran stripped serum (HyClone). Estradiol (17β-estradiol) and 4-OH-tamoxifen were purchased from Sigma (St. Louis, MO). The 27-18 cell line (Gao *et al.*, 1996) was derived from HEK293 cells after stable transfection with a plasmid expressing adenovirus E4 ORF 6 under the control of a mouse mammary tumor virus (MMTV) promoter. These cells were maintained in DMEM plus 10% fetal bovine serum and G418 (1 mg/ml; GIBCO-BRL). The expression of the E4 ORF 6 in these cells was induced by adding 10 μM dexamethasone (Gensia, Irvine, CA) to the culture medium. All culture media were supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and amphotericin B (Fungizone, 0.25 μg/ml; BioWhittaker).

Construction and characterization reporter plasmids

The estrogen-responsive promoter (EREp) was obtained from the plasmid pERE2pS2CAT (Montano *et al.*, 1996), kindly provided by B.S. Katzenellenbogen (University of Illinois, Urbana, IL). This plasmid contains a portion of the pS2 promoter (−90/+10 bp), plus two EREs. The fragment containing the promoter was liberated by digestion with *Eco*RI and *Bsr*I, and subcloned in the *Sma*I site of the luciferase reporter plasmid pGL2-Basic (Promega, Madison, WI). To verify the

response to estrogens, the new construct (pBERE) was transfected into T47D, MCF7, or MDAMB231 cells using the FuGENE 6 reagent, as described by the manufacturer (Roche, Indianapolis, IN). The cells (typically 7.5×10^4/well in a 12-well plate) were seeded in estrogen-free medium and transfected 24 hr later, using 0.3 μg of pBERE and 0.1 μg of the plasmid pRLTK (Promega) in order to quantify the efficiency of transfection. To confirm the specificity of transcriptional activation, a promoterless reporter (pGL2-Basic) and a reporter with a constitutive simian virus 40 (SV40) promoter (pGL2-Control) were transfected under the same conditions. Six hours later, the transfection medium was removed and new medium was added containing the indicated treatments. After 14–18 hr, cells were lysed and analyzed for luciferase activity with the dual luciferase reporter assay system (Promega), as indicated by the manufacturer. The specific luciferase activity measured in a luminometer is presented as (firefly/*Renilla*) $\times$ 1000 luciferase units.

Construction of Ad5ERE2

Modification of the adenoviral genome. To modify the regions containing the E1a and E4 promoters, we subcloned portions of the adenovirus type 5 genome in plasmids that allowed their manipulation by standard cloning techniques. For the E4 region, the *Eco*RI–*Bam*HI fragment of the pAdTrack plasmid (kindly provided by B. Vogelstein, Howard Huges Medical Institute, Baltimore, MD) containing nucleotides 34931 to 35935 of the viral genome (He *et al.*, 1998), was subcloned into the pUC19 vector (New England BioLabs, Beverly, MA). The new plasmid was called pUC19Track. To eliminate the E4 promoter, a deletion comprising nucleotides 35575 to 35818 was performed. To do this, the viral region comprising nucleotides 35233 to 35575 was amplified by polymerase chain reaction (PCR) using primers that introduced an *Nru*I site at position 35575. The primers were 5′-AAACTGGTCACCGTGATTAAAAAG-3′ and 5′-CCGTAGGATTCACCATCAGGTGTCGCGACGAGTGGTGTTTTTTTAA-3′. The latter primer contains a 22-bp random sequence at the 5′ end. In a similar way, the region comprising nucleotides 35818 to 35935 was amplified with primers that introduced an *Ecl*XI site at position 35818, and produced a 22-bp sequence complementary to the previous one. These primers were 5′-CACCTGATGGTGAATCCTACGGCGGCGGCCGACTCCGCCCTAAAACC-3′ and 5′-CGATCATTAATTAACATCATCAATAATAATATACC-3′. The products obtained with these sets of primers were combined to obtain a fragment in which the E4 promoter is substituted by the complementary random sequence flanked by *Nru*I and *Ecl*XI sites.

This fragment was amplified by PCR using the most external primers of the previous reactions, and was introduced in the *Bst*E_2–*Pac*I sites of pUC19Track. We then engineered a second plasmid (pShutAd) containing the kanamycin resistance gene from pShuttle (He *et al.*, 1998), the deleted E4 promoter region, and the E1a promoter-containing adenoviral sequence from nucleotides 1 to 1574 subcloned from the pTG3602 plasmid (Chartier *et al.*, 1996), kindly provided by M. Mehtali (Transgène, Strasbourg, France). On the basis of pShutAd, we could introduce the ERE2 promoter into the E4 region, (*Nru*I–*Ecl*XI sites, blunt ended). The deletion of the E1a promoter (nucleotides 341 to 499) was obtained by combination of two PCR fragments, following the same strategy that led to the deletion of the E4 promoter. The fragment comprising nucleotides 341 to 499 was amplified with the primers 5′-AGCGCGTAATATTGAATTCGGGCCGCGGGGACTTTGA-3′ and 5′-TCTACTCGCTGGCACTCAAGAGTCGCGACTTGAGGAACTCAC-3′. These primers incorporate *Eco*RI and *Nru*I sites flanking the E1a promoter, and a complementary tail to the fragment produced in the second PCR. The latter amplified the region comprising nucleotides 499 to 1339. The primers used were 5′-CTCTTGAGTGCCAGCGAGTAGA-3′ and 5′-TGCATTCTCTAGACACAGGTGATGTC-3′. Once the two portions were fused and amplified, the product was subcloned in the *Ssp*I–*Xba*I sites of pShutAd, and the E1a promoter could be substituted by the EREp promoter (*Nru*I–*Eco*RI sites, blunt ended). Once the modifications of the E1 and E4 regions were completed, we used homologous recombination in *Escherichia coli* to introduce these changes in the whole adenovirus genome. The modified plasmid confers kanamycin resistance to the cells. It was cotransformed in BJ5183 cells with pTG3602, a plasmid that contains the wild-type adenovirus genome subcloned in a backbone with the ampicillin resistance genes. After electroporation, the cells were selected in LB plates containing kanamycin (30 μg/ml) and small colonies were screened by restriction endonuclease digestion. The DNA was purified from a single positive colony, and confirmed by automated sequencing of the modified regions.

Production and verification of the virus. Once the plasmid containing the modified adenovirus was obtained, it was digested with *Pac*I to liberate the viral genome from the plasmid backbone. After phenol–chloroform extraction and ethanol precipitation, the DNA was transfected into 27-18 cells by the LipofectAMINE method (Gibco-BRL), as described by the manufacturer. The cells were maintained in DMEM plus 5% fetal bovine serum (FBS) and 10 μM dexamethasone to induce the expression of the E4 ORF 6. When the cytophatic effect was evident in the monolayer (typically 6–8 days after transfection), the cells were collected and lysed by three rounds of freezing and thawing in phosphate-buffered saline (PBS) supplemented with 0.01% $CaCl_2$ and 0.01% $MgCl_2$ (PBS^{++}). After spinning the lysate at $350 \times g$ to discard the cellular debris, the supernatant was used to infect a monolayer of 27-18 cells. Viral plaques were isolated from this monolayer, and the viral DNA was extracted to confirm the modifications by PCR. The supernatant containing viruses was digested for 2 hr with autodigested pronase (0.5 μg/ml) in a buffer containing 10 mM Tris, pH 7.5, and the DNA was phenol–chloroform purified and ethanol precipitated. Twenty nanograms was used in the PCRs. The region containing the E1 promoter was amplified with the primers 5′-TAGTGTGGCGGAAGTGTGATGTTG-3′ (complementary to nucleotides 104 to 128 of the adenovirus sequence) and 5′-TCTTCGGTAATAACACCTCCGTGG-3′ (complementary to nucleotides 577 to 600). The region containing the E4 promoter was amplified with the primers 5′-AAACTGGTCACCGTGATTAAAAAG-3′ (complementary to nucleotides 35233 to 35251) and 5′-CCGTAGGATTCACCATCAGGTGTCGCGACGAGTGGTGTTTTTTTAA-3′ (complementary to the end of the adenoviral genome). The PCR fragments were digested with *Cla*I, a restriction endonuclease

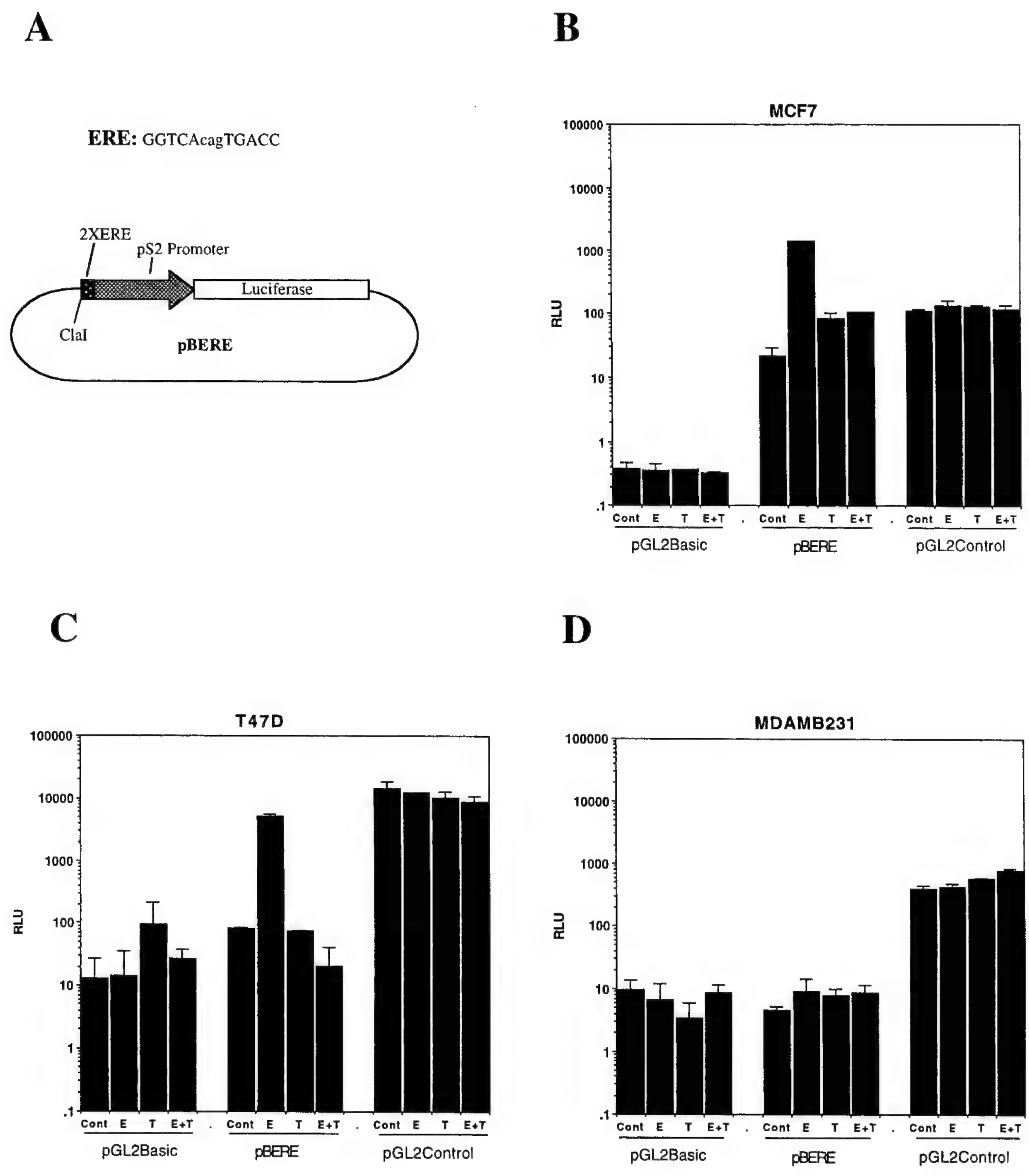


FIG. 1. Construction and characterization of the estrogen-inducible promoter (EREp). (**A**) Schematic representation of pBERE, a reporter plasmid expressing the luciferase gene under the control of the EREp promoter. The ERE consensus sequence is indicated. The gray arrow represents the pS2 promoter fragment (nucleotides −90/+10). Two EREs are subcloned upstream of this region (dark dotted box), and the position of the *Cla*I site is indicated. (**B**) ER^+ MCF7 human breast cancer cells were transfected with 0.3 μg of either pGL2Basic, pBERE, or pGL2Control reporter plasmids, together with 0.1 μg of the pRLTK *Renilla* luciferase plasmid to correct for efficiency of transfection. Phenol red-free medium and 2.5% charcoal-dextran stripped serum were used. After 6 hr, the medium was changed and cells were left untreated (Cont), or treated with 2.5 n*M* 17β-estradiol (E), 2.5 μM 4-OH-tamoxifen (T), or both (E + T), as indicated on the horizontal axis. Sixteen hours later, cells were lysed and both firefly and *Renilla* luciferase activities were measured in protein extracts. The columns represent in logarithmic scale the relative luciferase units (RLU) of (firefly/*Renilla*) × 1000. (**C** and **D**) The results of the same kind of assay performed in the ER^+ T47D and ER^- MDAMB231 human breast cancer cell lines, respectively. The assay was repeated three times with similar results.

site that is present in the EREp promoter, but not in this region of the wild-type virus. Large-scale preparations of the virus were produced in 27-18 cells and purified by cesium chloride centrifugation. Titrations were made by plaque forming assay, and multiplicity of infection (MOI) refers to plaque-forming units (PFU) per number of cells.

Analysis of transcriptional activation of E1a and E4 units

To analyze the responsiveness of the EREp promoter in the context of the adenoviral genome, we performed Northern blot assays of cells infected with Ad5ERE2. MCF7 cells (2×10^6 cells/60-mm plate) were pretreated for 12 hr in estrogen-free medium. Infection was performed for 1 hr in 1 ml of PBS^{++}. The infection medium was then removed and the cells were incubated for 9 hr in the treatment medium. Total RNA was extracted with the Trizol reagent (GIBCO-BRL), and 10 μg of RNA was formaldehyde–formamide denatured, fractionated in a 1.2% agarose gel with 2.2 *M* formamide, and transferred to a nylon membrane (Hybond-N+; Amersham, Buckinghamshire, UK). The E1a probe consisted of the 998-bp *Ssp*I–*Xba*I fragment from the adenoviral genome (nucleotides 341 to 1339), and the E4 probe was the 800-bp *Ssp*I–*Asn*I fragment (nucleotides 34634 to 35419). They were radiolabeled by use of a random primed DNA labeling kit (Roche). The membrane was prehybridized for 2 hr at 68°C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) in the presence of salmon sperm DNA (1 mg/ml) and tRNA (1 mg/ml). Hybridization was at 68°C for 1 hr with probe at 2×10^6 cpm/ml. The membrane was then washed with $2 \times$ SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 min, and twice with $0.1 \times$ SSC, 0.1% SDS at 50°C ($1 \times$ SSC is 0.15 *M* NaCl and 15 m*M* sodium citrate, pH 7.0). Finally, it was autoradiographed. To confirm the homogeneous RNA loading and hybridization, we used the same membranes and hybridized them with a mouse β-actin probe.

Cytopathic effect and viability of cells

After infection of the cell lines with a wild-type adenovirus (Ad5WT) or Ad5ERE2, the appearance of cytopathic effect (rounding and detachment) was monitored and cells were photographed with a digital camera (Pixera, Los Gatos, CA). To quantify the viability of cells, the MTT assay was used (Sigma). At the time of analysis, 25 μl of thiazolyl blue (MTT, 5 mg/ml) solution was added to the cells cultured in 100 μl of medium (96-well plate). Five hours later, 100 μl of solubilization solution (20% [w/v] SDS in 50% [v/v] *N,N*-dimethylformamide) was added, and 12 hr later the absorbance at 600 nm was read in a spectrophotometer. When the experiment was performed in 12-well plates, cells were washed with 1 ml of PBS before adding the MTT reagent (250 μl for 1 ml of culture medium/well), and 1 ml of solubilization solution was added 5 hr later. The significance of the differences observed was analyzed with the *t* test.

X-Gal staining of cells in culture

To analyze the complementation of the E1a deficiency of adenoviral vectors with Ad5ERE2, an E1a-deleted vector expressing the β-galactosidase (*lacZ*) gene, Ad5LacZ, (Ealovenga *et al.*, 1996), was coinfected with Ad5ERE2. The cells infected with Ad5LacZ can be identified in the monolayer by fixing and staining with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), which produces a blue precipitate on hydrolysis by the enzyme β-galactosidase. The monolayers were fixed for 5 min in a solution containing 5.4% (w/w) formaldehyde and 0.8% (w/w) glutaraldehyde in PBS. The fixative was then removed and the cells were covered with staining solution and incubated at 37°C until the blue precipitate appeared. The staining solution contained X-Gal (1 mg/ml; GIBCO-BRL), 2 m*M* $MgCl_2$, 5 m*M* $K_3Fe(CN)_6$, and 5 m*M* $K_4Fe(CN)_6$ in PBS. Finally, the number of blue cells per field was counted in duplicate wells.

In vivo *assays in nude mice*

The antitumor effect of Ad5ERE2 was tested in human tumor xenografts implanted in 5- to 7-week-old BALB/c *nu/nu* mice (Harlan, Indianapolis, IN). Estradiol pellets (0.72 mg of 17β-estradiol, 60-day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the mice. Three days later, 5×10^6 MCF7 cells were resuspended in serum-free RPMI plus 20% Matrigel (Collaborative Biomedical Products, Bedford, MA) and injected subcutaneously in the back flanks. T47D cells were resuspended in PBS and injected in the mammary fat pad. When tumors reached a volume of approximately 100 mm^3, viruses were diluted in 50 μl of PBS and injected intratumorally with a Hamilton syringe.

RESULTS

Construction and characterization of reporter plasmids containing a promoter that responds to estrogen in ER^+ cells and can be inhibited by tamoxifen

To obtain a promoter that would be preferentially active in breast cancer cells, we used a construct (pBERE) containing a fragment of the human pS2 promoter (−90/+10 bp), with two EREs that confer its estrogen responsiveness (Berry *et al.*, 1989). The pS2 protein is a member of the trefoil factors, which are normally expressed in the mucosa of the gastrointestinal tract (Ribieras *et al.*, 1998). In addition, approximately 50% of human breast cancers show ectopic expression of pS2/TFF1 (Rio and Chambon, 1990), generally in estrogen receptor-positive (ER^+) cases. In fact, pS2 levels increase after estrogen treatment in the human breast cancer cell line MCF7 (Jeltsch *et al.*, 1987). The combination of the pS2 5′-flanking sequence and the EREs ensures a strong activation of the promoter in response to estrogens, only in ER^+ cells (Ekena *et al.*, 1998). To characterize the promoter in our *in vitro* system, we subcloned it into a luciferase reporter plasmid (pGL2-Basic), and analyzed the activation by 17β-estradiol. Figure 1A shows the structure of the plasmid containing the luciferase gene under the control of the estrogen responsive promoter (pBERE). Two different ER^+ human breast cancer cell lines (MCF7 and T47D) were transfected with the pBERE plasmid in a culture medium depleted of estrogens, and some wells were treated with 17β-estradiol as indicated in Fig. 1B and C. Both cell lines responded to estrogens with more than 50-fold induction. When the cells were treated with the antiestrogen 4-OH-tamoxifen, the estro-

genic stimulation was abolished. To confirm the specificity of the activation, we transfected the cells with two additional luciferase reporter plasmids. pGL2-Basic is a promoterless plasmid and pGL2-Control has the luciferase gene under the control of a constitutive SV40 promoter. When these plasmids were used, there was neither significant increase of luciferase activity after treatment with estrogen, nor inhibition with 4-OH-tamoxifen. When the same experiment was performed with the ER^- breast cancer cell line MDAMB231, no increase in luciferase activity was observed in cells transfected with the pBERE plasmid (Fig. 1D). These data indicate that the EREp promoter can be modulated by estrogenic agonists/antagonists only in cells that express ERs. An increase in luciferase activity was observed in untreated ER^+ cells transfected with pBERE compared with the promoterless plasmid. The effect was more evident in MCF7 cells. This apparently unspecific activation can be due in part to the traces of estrogens present in the culture medium, because it was not observed in the ER^- cell line MDAMB231.

Construction of Ad5ER2, an adenovirus with E1a and E4 regions controlled by the EREp promoter

The data presented in Fig. 1 indicate that the EREp promoter is suitable for directing the expression of a gene to ER^+ cells. To obtain an adenovirus that will replicate preferentially in this

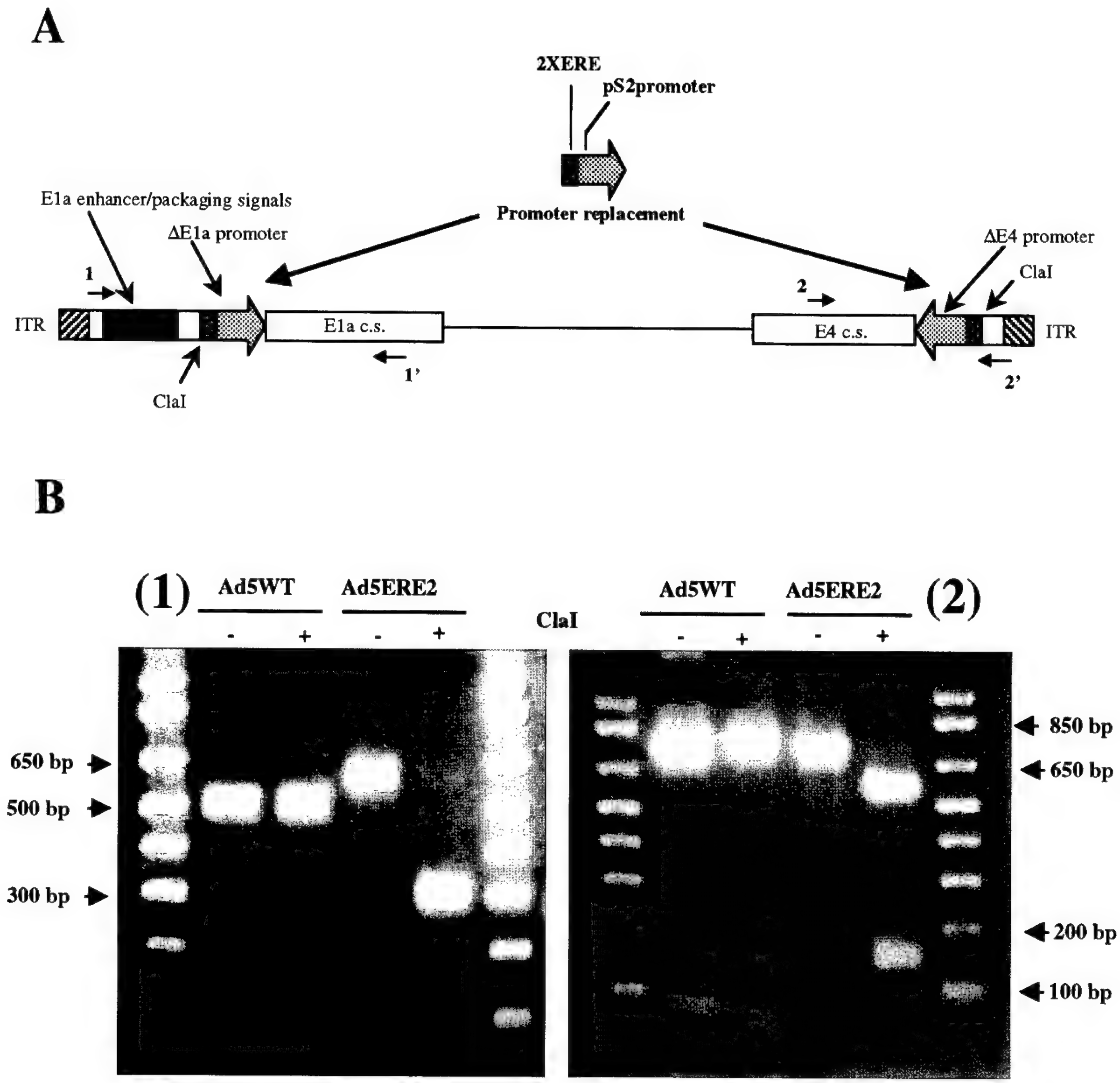


FIG. 2. Construction of Ad5ERE2. (**A**) Schematic representation of the modifications introduced in the adenovirus type 5 genome. The size of the viral regions is not proportional in the scheme. At the left end of the genome, the E1a enhancer/packaging signal is represented as a black box. The deleted E1a and E4 promoters are replaced by the EREp promoter. The positions of the PCR primers used to verify the structure of the virus are indicated as small arrows. (**B**) DNA was extracted from Ad5WT and Ad5ERE2, and PCRs were performed with primers 1 and 1′ (panel 1), or primers 2 and 2′ (panel 2). The + sign indicates digestion of the PCR fragment with the restriction endonuclease *Cla*I. The fragments were resolved in a 1.5% agarose gel.

kind of cell, we placed the E1a and E4 regions of the virus under the control of this promoter. The wild-type promoters were deleted by a PCR strategy as described in Materials and Methods. The deletions comprise nucleotides 35575 to 35818 in the E4 promoter, and nucleotides 341 to 499 in the E1a promoter. Figure 2A shows a schematic representation of the modifications introduced in the sequence of the adenovirus type 5. This modified sequence was used to generate a new virus called Ad5ERE2, as described in Materials and Methods. The viral DNA was isolated and the modifications were confirmed by PCR (Fig. 2). In the E1a region, the wild-type adenoviral DNA yields a fragment of 500 bp, whereas the Ad5ERE2 fragment is 570 bp. In the case of the E4 region, the difference in size between both viruses is not as marked (700 bp for wild type and 680 bp for Ad5ERE2). To verify further the presence of the EREp promoter and eliminate the possibility of contamination with wild-type virus, the fragments were isolated and digested with *Cla*I, a restriction enzyme whose recognition sequence is present in the EREp, but not in the wild-type promoter. As shown in Fig. 2, *Cla*I excised the PCR fragments of Ad5ERE2, but not the wild-type adenovirus.

The function of a promoter can be drastically affected by the presence of other regulatory elements, especially in the case of a complex viral genome (Shi *et al.*, 1997; Vassaux *et al.*, 1999). Therefore, we determined whether the EREp promoter is able to regulate the expression of the E1a and E4 units in the context of the adenoviral genome. To do this, we infected monolayers of MCF7 cells with Ad5ERE2 at an MOI of 5 virus/cell in the absence or presence of estrogens. Nine hours after infection, total RNA was extracted and the transcriptional activation of E1a and E4 regions was analyzed by Northern blot. There is a strong activation of the E1a and E4 mRNAs after estrogen treatment in this breast cancer cell line, which is drastically inhibited by the concomitant addition of the antiestrogen 4-OH-tamoxifen (Fig. 3). These data indicate that the E1a and E4 transcription units can be activated by an exogenous promoter after the deletion of the wild-type activator. In addition, the ERE2 promoter retained its ability to respond to estrogens.

The cytopathic effect of Ad5ERE2 can be modulated by estrogens and tamoxifen, and correlates with the expression of the estrogen receptors in different cell lines

Once we had verified the modulation of E1a and E4 viral gene expression in response to estrogenic agonists/antagonists, we investigated the capacity of the virus to induce cytopathic effect and death of the target cells. MCF7 cells were infected with Ad5ERE2 at an MOI of 1 PFU/cell for 8 days. Some cell monolayers were maintained in estrogen-free medium, whereas others were treated with 17β-estradiol, alone or in combination with tamoxifen, as indicated in Fig. 4. Eight days after infection, the viability of the cells was analyzed by the MTT assay (Carmichael *et al.*, 1987). The result showed that the addition of 17β-estradiol to MCF7 cells infected with Ad5ERE2 drastically increases the capacity of the virus to kill the cells. The viability of the cells infected in the absence of estradiol decreased only 25% compared with the noninfected cells growing under the same conditions after 8 days. On the other hand, when estradiol was added, virtually all the cells died, and tamoxifen partially inhibited this effect and allowed the survival of more than 50% of the cells.

To analyze if the modifications introduced in the virus affected its capacity to cause CPE and death of the cells in correlation with their ER status, we compared the effect of Ad5WT and Ad5ERE2 in six representative human cancer cell lines. Five of them (MCF7, T47D, MDAMB231, BT474, and SKBR3) are derived from breast cancer, whereas HeLa is an ER^- cell line derived from a cervical cancer, which shows high permissivity for infection with the wild-type adenovirus. The cells were infected in the presence of 17β-estradiol in the culture medium. Figure 5A and B shows microphotographs of representative $\times 100$ magnification fields. In Fig. 5A, the ER^+ cell lines MCF7 and T47D, and the ER^- cell lines MDAMB231 and HeLa, were infected at multiplicity of infection (MOI) of 5 virus/cell, and the monolayers were photographed 4 days after infection. In Fig. 5B, the ER^+ cell line BT474 and the ER^- cell line SKBR3 were infected at an MOI of 3 virus/cell, and were photographed 7 days after infection. All cell lines presented evident CPE when infected with Ad5WT, with disruption of the monolayer, rounding and detaching of cells. Ad5ERE2 caused similar changes in ER^+ cells. In contrast, ER^- cells were more resistant to the effects of this virus, both at early (Fig. 5A) and late (Fig. 5B) times after infection. This demonstrates that the mutations introduced in the viral genome are compatible with efficient viral production, and that the regulation of the E1a and E4 transcription units has the desired effect on the cytotoxicity of the virus. To better quantify this effect, we again used the MTT assay to measure viability after

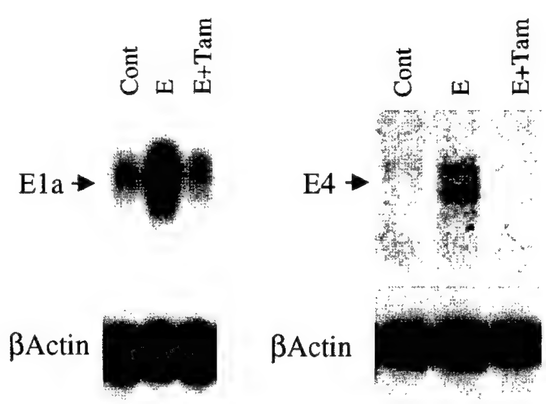


FIG. 3. Analysis of E1a and E4 transcription in Ad5ERE2-infected MCF7 cells. MCF7 cells were maintained in phenol red-free medium with 2.5% charcoal–dextran stripped serum, and left untreated (Cont) or pretreated for 12 hr with 2.5 nM 17β-estradiol (E) or 2.5 nM 17β-estradiol plus 2.5 μM 4-OH-tamoxifen (E + Tam) as indicated above each lane. Cells were then infected with Ad5ERE2 at an MOI of 5 virus/cell and total RNA was extracted after 9 hr of infection. Northern blot analysis was performed with radioactive probes specific for the E1a, E4 regions or the constitutively expressed β-actin gene as a control.

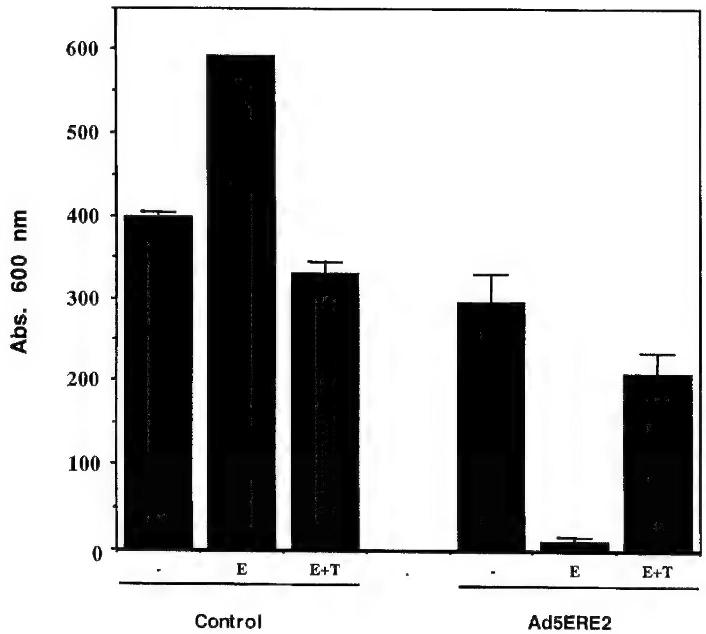


FIG. 4. Modulation of the cytotoxicity of Ad5ERE2 with estrogenic agonists/antagonists. MCF7 cells were maintained in phenol red-free medium with 2.5% charcoal–dextran stripped serum, and left untreated (–) or treated with 2 n*M* 17β-estradiol (E) or 2 n*M* 17β-estradiol plus 2.5 μM 4-OH-tamoxifen (E + T). One set of cells was infected with Ad5ERE2 at an MOI of 1 virus/cell, and another set was left uninfected (Control), as indicated on the horizontal axis. The monolayers were refed every 3 days, and 8 days after initiation of the infection the viability of the cells was analyzed by the MTT assay. The vertical axis indicates the absorbance at 600 nm, which is proportional to the number of surviving cells.

infecting the cells with Ad5WT or Ad5ERE2 at an MOI of 3 virus/cell in the presence of 17β-estradiol. Cells were analyzed 3 days (Fig. 6A) or 7 days (Fig. 6B) after infection. In agreement with the CPE assay described above, Ad5ERE2 reduced the viability of MCF7, T47D, and BT474 cells as efficiently as Ad5WT ($p > 0.05$). On the other hand, MDAMB231, HeLa, and SKBR3 cells were at least two times more resistant to the cytotoxic effect of Ad5ERE2 compared with the wild-type virus. The result of this assay indicates that Ad5ERE2 is fully active in ER^+ cells, whereas there is a significant ($p < 0.001$) attenuation of the cytotoxic effect in the ER^- cells.

Finally, we analyzed the effect of Ad5ERE2 on primary cells derived from a human breast cancer. The ER^+ SUM-309PE cells were infected at a low MOI (2 virus/cell), in order to test if this primary cell line can sustain the proliferation of the virus. The morphology and the viability of the monolayers were monitored 3, 5, and 8 days after infection. After 3 days, a small portion of cells started to show cytopathic effect, but the reduction of viability was modest (Fig. 7A and B). After 5 days, the cytopathic effect was widespread in the monolayer, and the viability was reduced 50% compared with the uninfected cells. Three days later, the reduction was more than 90%, which suggest that Ad5ERE2 can efficiently infect, replicate in, and kill primary breast cancer cells.

Use of Ad5ERE2 to allow replication of an E1-deleted adenovirus

Apart from being a therapeutic tool itself, Ad5ERE2 can be used to complement the E1a deficiency of already existing replication-incompetent adenoviral vectors. There are several E1a-deleted adenoviruses engineered to deliver lethal genes to cancer cells. Although they can efficiently deliver the gene and obtain high levels of expression that lead to the death of the infected cells, the cells that escape the initial infection are not affected and cause the relapse of the tumor.

The expression of E1a proteins in Ad5ERE2 is controlled by a promoter that is preferentially activated in ER^+ cells. This means that if a cell is coinfected with Ad5ERE2 and an E1a-deleted adenovirus, the same regulation of replication can apply to both viruses because Ad5ERE2 will provide the E1a protein in *trans.* To do this, we used an E1a-deleted adenovirus vector carrying the β-galactosidase gene under the control of a constitutive Rous sarcoma virus (RSV) promoter (Ad5LacZ), which allowed a quantification of the complementation. T47D cells were infected with a suboptimal amount of Ad5LacZ (MOI of 5), alone or in combination with Ad5ERE2 (MOI of 1). The infection was performed in estrogen-depleted medium and duplicate wells were left untreated or were treated with 17β-estradiol or 17β-estradiol plus tamoxifen. Five days later, the monolayers were fixed and incubated with the substrate X-Gal to identify cells that were infected with Ad5LacZ. The average number of blue cells per field was counted and the results are graphically represented in Fig. 8. Surprisingly, when Ad5LacZ was used alone, the addition of estrogens reduced slightly but significantly the number of positive cells, and this effect was reversed by tamoxifen. Importantly, the addition of Ad5ERE2 stimulated more than four times the number of cells infected by Ad5LacZ under these conditions. The cooperation is mediated by the regulated expression of the E1a protein under the

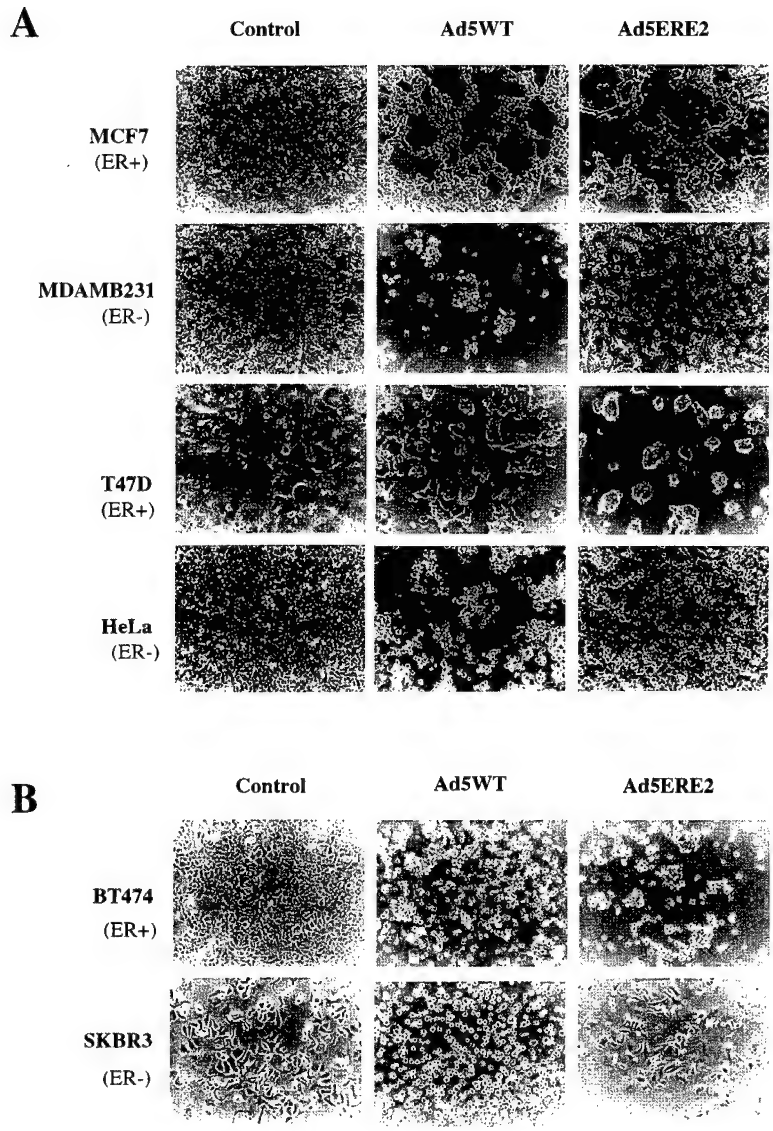


FIG. 5. Cytopathic effect of Ad5ERE2 on different cell lines. (**A**) The ER^+ cell lines MCF7 and T47D, and the ER^- cell lines MDAMB231 and HeLa, were infected with Ad5WT or Ad5ERE2 at an MOI of 5 virus/cell in the presence of 1.5 n*M* 17β-estradiol. The monolayers were photographed 4 days after infection. Representative fields (original magnification, $\times 100$) are shown. The control panels are noninfected cells maintained under identical conditions. The assay was repeated four times with similar results. (**B**) The same assay was performed with the ER^+ cell line BT474 and the ER^- cell line SKBR3. Cells were infected for 7 days at an MOI of 3 virus/cell.

control of the EREp promoter, because tamoxifen inhibited the complementation. The preceding results suggest that a controlled replication of an E1-deleted adenovirus can be obtained by supplementation of the E1a protein with a conditionally replicative adenovirus. Next, we determined whether this strategy could be used to amplify the killing capacity of an E1a-deleted adenovirus vector that expresses the proapoptotic gene Bcl-xs (Ad5Bcl-xs). It has been previously demonstrated that this virus can induce apoptosis in MCF7 cells in culture, and it inhibits the growth of tumors induced by injection of these cells in nude mice (Clarke *et al.*, 1995; Ealovenga *et al.*, 1996). However, the inhibitory effect was modest and frequently administrations of the virus were required. The addition of a conditionally replicative adenovirus such as Ad5ERE2 could enhance the cytotoxic effect of Ad5Bcl-xs. To test this, we infected MCF7 cells with Ad5Bcl-xs at MOIs of 2 and 20 virus/cell, which is an amount of virus 5000–500 times lower than the dose needed to effectively kill these cells in culture. Additional monolayers were infected with Ad5Bcl-xs plus Ad5ERE2 at an MOI of 0.5 virus/cell in the presence of 17β-estradiol, with or without tamoxifen. Neither Ad5Bcl-xs nor Ad5ERE2 alone showed significant cytotoxicity when used at these low titers.

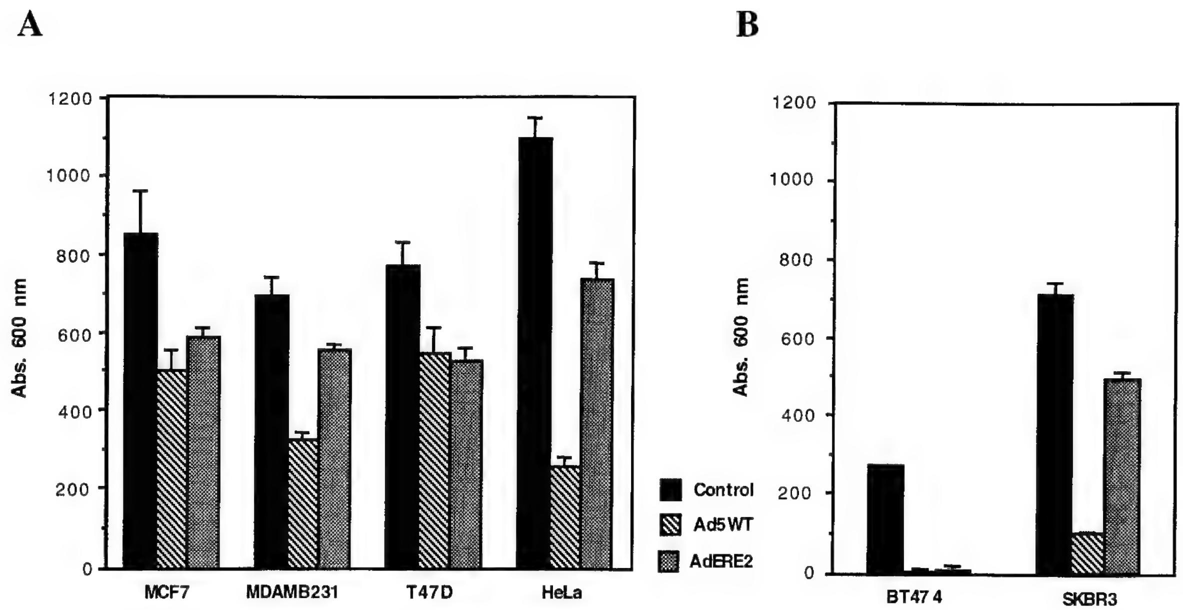


FIG. 6. Viability of various human cancer cell lines infected with Ad5ERE2. (**A**) The ER^+ cell lines MCF7 and T47D, and the ER^- cell lines MDAMB231 and HeLa, were infected with Ad5WT (striped columns) or with Ad5ERE2 (gray columns) at an MOI of 3 virus/cell in the presence of 1.5 n*M* 17β-estradiol. The viability of the cells was analyzed 3 days after infection by the MTT method. (**B**) The ER^+ cell line BT474 and the ER^- cell line SKBR3 were infected as indicated above, and the viability was analyzed 7 days after infection.

The cells were refed every 3 days, and the viability was analyzed 8 days after infection. As shown in Fig. 9A, Ad5ERE2 greatly enhanced the killing effect of Ad5Bcl-xs. Again, the complementation elicited by Ad5ERE2 can be specifically inhibited with the antiestrogen tamoxifen. In Fig. 9B, the cells were treated basically as described above, but their viability was analyzed after 8 days of culture by the MTT assay. Ad5Bcl-xs was used at a higher MOI (200 virus/cell), and the MOI of Ad5ERE2 was as low as 0.25. The result indicates that this concentration of Ad5Bcl-xs was not lethal for MCF7 cells, whereas Ad5ERE2 caused a slight ($p = 0.07$) decrease in viability. However, when both viruses were combined, 90% of the cells died at this time point. The addition of tamoxifen caused a significant ($p < 0.005$) protection from the lethal effect of this combination of viruses. In addition, a control E1a-deleted adenovirus without exogenous gene expression (Ad5ΔE1a) was coinfected with Ad5ERE2 under the same conditions. In contrast with Ad5Bcl-xs, no significant ($p = 0.08$) decrease in the viability of the cells was observed with this combination. This indicates that the amplification of the killing effect of Ad5Bcl-xs depends on the expression of the proapoptotic gene, and is not only the consequence of the replication of the vector. These results suggest that the effect of E1a-deleted adenoviral vectors can be amplified in a controlled fashion by using a replication-conditional adenovirus that supplements the E1a protein.

Antitumor effect in vivo

The *in vivo* antitumor effect of Ad5Bcl-xs has already been demonstrated (Ealovenga *et al.*, 1996), but the limitations of the first generation of adenoviral vectors to deliver genes *in vivo* markedly reduces their utility. We observed no significant inhibition of MCF7-induced tumor growth in nude mice when the virus was administered at 10^8 PFU/tumor once a week instead of twice a week (data not shown). Therefore, we tested the antitumor effect of the combination of Ad5Bcl-xs and Ad5ERE2 on human tumor xenografts under the same conditions. Tumors were induced by injection of MCF7 or T47D cells subcutaneously in estrogen-treated mice. The viruses were administered intratumorally, once a week for 3 weeks. The data presented in Fig. 10 correspond to the percentage of reduction of tumor volume 20 days after the first viral administration. For MCF7 tumors, Ad5ERE2 was used at 10^7 PFU/tumor, alone or in combination with Ad5Bcl-xs, 10^8 PFU/tumor. For T47D tumors, the dose of Ad5ERE2 was 5×10^7 PFU/tumor. In both tumors, Ad5ERE2 was able to reduce the average tumor volume more than 50%, but only the combination of Ad5ERE2 and Ad5Bcl-xs obtained a drastic reduction of more than 90% (Fig. 10), much higher than the effect for five similar doses of Ad5Bcl-xs alone (data not shown).

DISCUSSION

The use of tumor-specific promoters to control the expression of viral genes is a promising method for the construction of therapeutic viruses. The lack of well-characterized promoters limits the systematic application of this technique. The systems to obtain drug-regulated gene expression constitute another new emerging field in gene therapy (Clarckson, 2000).

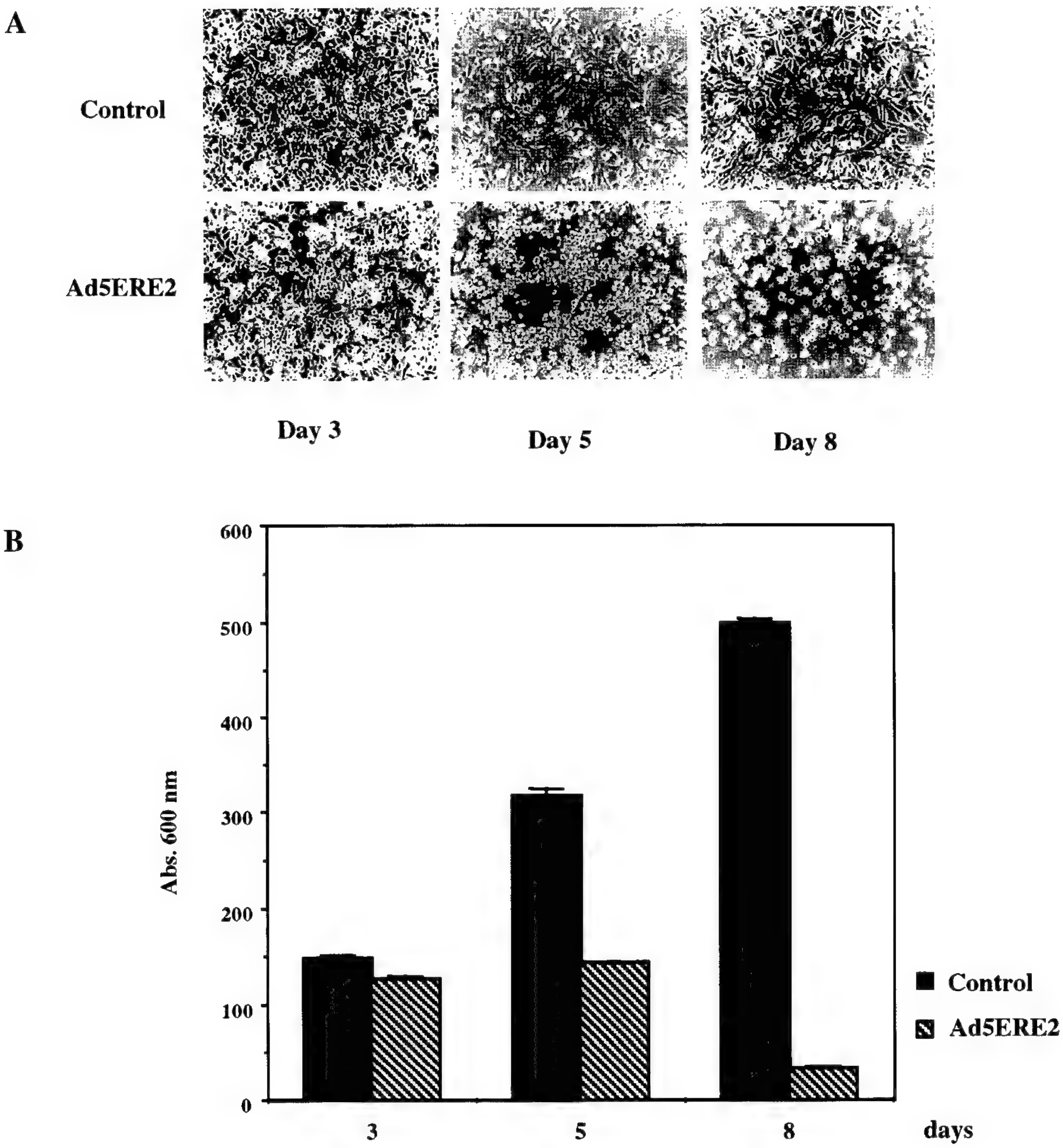


FIG. 7. Effect of Ad5ERE2 on primary human breast cancer cells. The ER^+ SUM-309PE cells were infected with Ad5ERE2 at an MOI of 2 virus/cell in the presence of 1.5 n*M* 17β-estradiol, and were refed every 3 days. (**A**) Cells were photographed 3, 5, and 8 days after infection. Control refers to uninfected cells photographed at the same time. Representative fields (original magnification, $\times$100) are shown. (**B**) The viability of the cells was analyzed by the MTT method.

We are interested in the development of a conditionally replicative adenovirus for the treatment of breast cancer. As an initial approach to direct the replication of the virus to breast epithelial cells in a controlled fashion, we used a promoter containing EREs. Only in cells that express ERs can the circulating estrogens activate this promoter. The use of EREs may also offer several advantages. First, this strategy allows the exogenous modulation of the virus by administration of antiestrogens such as tamoxifen, which is a well-tolerated drug extensively used in humans for the treatment and prevention of hormone-sensitive breast cancers. In addition, if a local or regional treatment is envisioned, the ERs can provide a preferential replication of the virus in malignant breast epithelial cells versus the surrounding parenchyma. Finally, although other tissues can express ERs, it is not clear that in all cases the levels and subtypes of receptors (ERα or ERβ) can stimulate viral replication.

In the Ad5ERE2 virus, two early transcription units (E1a and E4) are conditionally expressed. As both are necessary for an efficient viral replication, we believe that this contributes to the

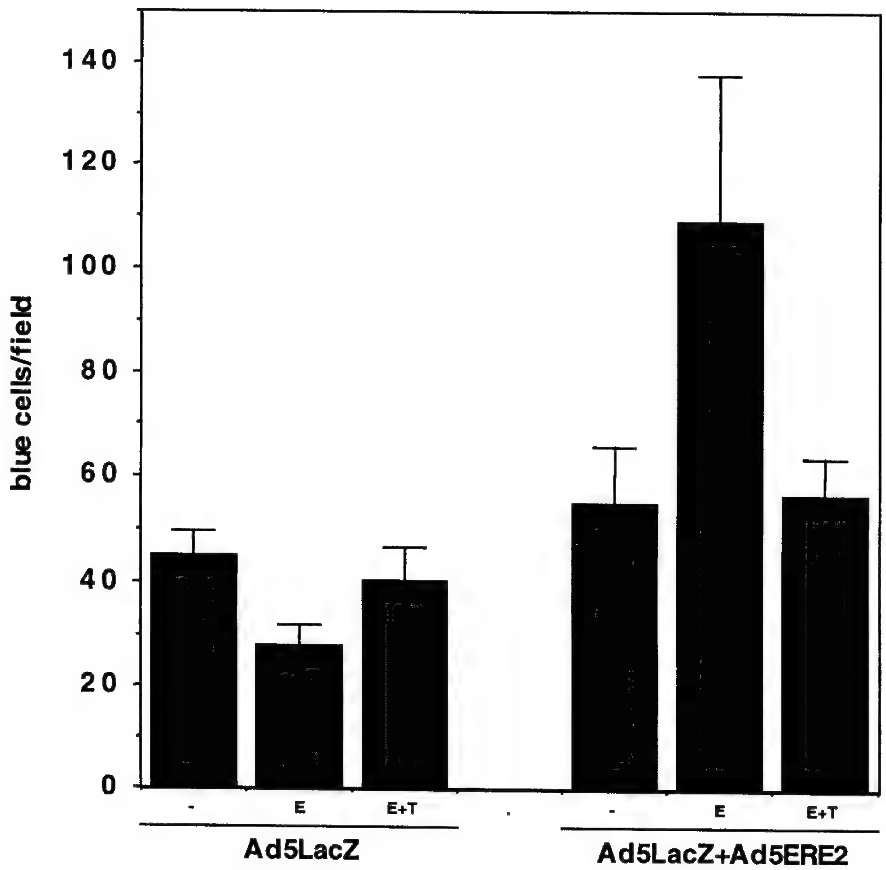
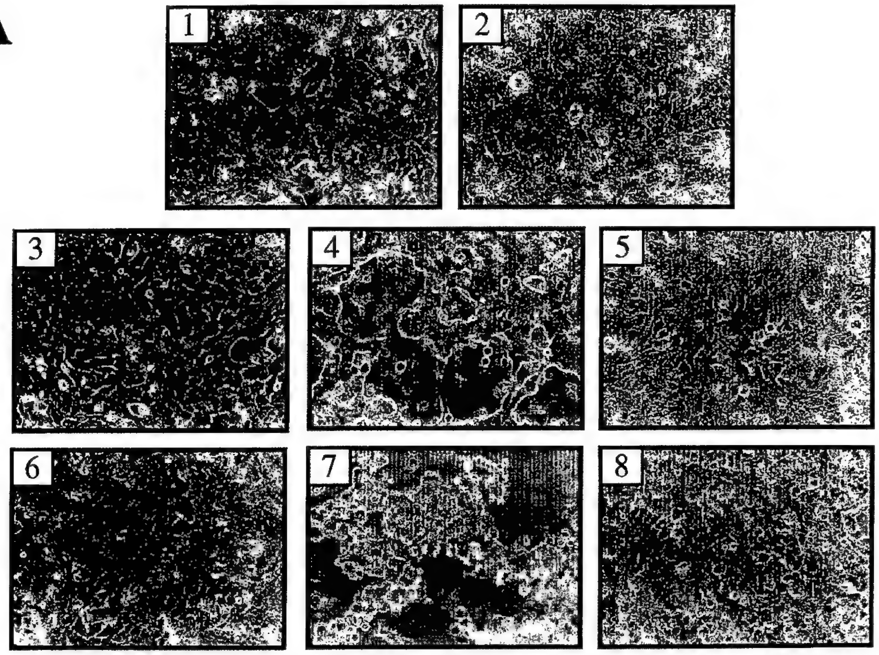


FIG. 8 Complementation of an E1a-deleted adenovirus vector with Ad5ERE2. T47D cells were grown in 12-well plates and infected with a suboptimal amount (MOI of 5 virus/cell) of a replication-deficient E1a-deleted adenoviral vector expressing the β-galactosidase gene (Ad5LacZ). The cells were maintained in phenol red-free medium with 2.5% charcoal–dextran stripped serum, and left untreated (–) or treated with 2 nM 17β-estradiol (E) or 2 nM 17β-estradiol plus 2.5 μM 4-OH-tamoxifen (E + T). Duplicate wells were infected with the same amount of Ad5LacZ plus Ad5ERE2 at an MOI of 1 virus/cell. Five days after infection, the monolayers were fixed and incubated with X-Gal as described in Materials and Methods. The number of Ad5LacZ-infected cells (blue cells) was counted in five different fields of each well. The assay was repeated two times with similar results.

control of the virus and constitutes a safety mechanism to prevent the appearance of wild-type revertants. Moreover, E4 functions have been implicated in virus-induced CPE and death of infected cells. In this way, the control of the E4 ORF expression can direct not only the replication, but also the lethal effect of the virus on the cancer cells, which is the final goal of therapeutic viruses. In the present study we demonstrate that the cytotoxic effect of the Ad5ERE2 virus can be modulated by the addition of estrogenic agonists/antagonists. More importantly, using several well-defined human tumor

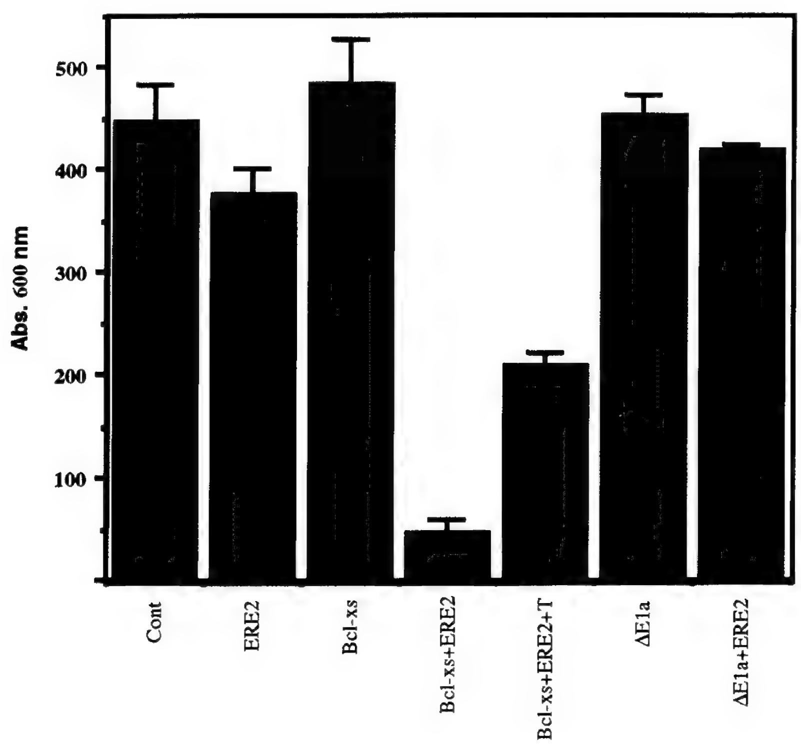
FIG. 9. Use of Ad5ERE2 to amplify the lethal effect of an E1a-deleted adenovirus vector expressing the proapoptotic gene Bcl-xs. (**A**) MCF7 cells (typically 5×10^4/well in a 12-well plate) were infected with suboptimal amounts of Ad5Bcl-xs (MOI of 2 and 20 virus/cell, as indicated), in the presence of 1.5 nM 17β-estradiol. In some of the monolayers, Ad5ERE2 was added at an MOI of 0.5 virus/cell. When indicated, 2 μM 4-OH-tamoxifen was added (T). Panel 1 shows uninfected cells, and panels 2 and 3 show cells infected with Ad5ERE2 alone (MOI of 0.5) or Ad5Bclx-s alone (MOI of 2), respectively. In panels 4 and 5, cells were infected with the indicated titers of both viruses together, with addition of tamoxifen only in panel 5. Panel 6 shows cells infected with Ad5Bcl-xs alone (MOI of 20). In panels 7 and 8, cells were infected with Ad5Bcl-xs (MOI of 20) plus Ad5ERE2 (MOI of 0.5), with addition of tamoxifen only in panel 8. The cells were refed with new medium every 3 days, and representative ×400 (original magnification) fields were photographed after 8 days of infection. The experiment was repeated three times with similar results. (**B**) In an independent experiment, MCF7 cells were treated essentially as described above. Ad5Bcl-xs was added at an MOI of 200 virus/cell, alone or in combination with Ad5ERE2 at an MOI of 0.25 virus/cell. In the same way, Ad5ΔE1a was used at an MOI of 200, alone or in combination with Ad5ERE2. After 8 days of infection, the viability of the cells was analyzed by the MTT assay, as described in Materials and Methods. The experiment was repeated two times with similar results. The significance of the differences observed was statistically analyzed by the t test.

A



1.- Control
2.- ERE2
3.- Bcl-xs MOI 2
4.- Bcl-xs MOI 2 + ERE2
5.- Bcl-xs MOI 2 + ERE2 + T
6.- Bcl-xs MOI 20
7.- Bcl-xs MOI 20 + ERE2
8.- Bcl-xs MOI 20 + ERE2 + T

B



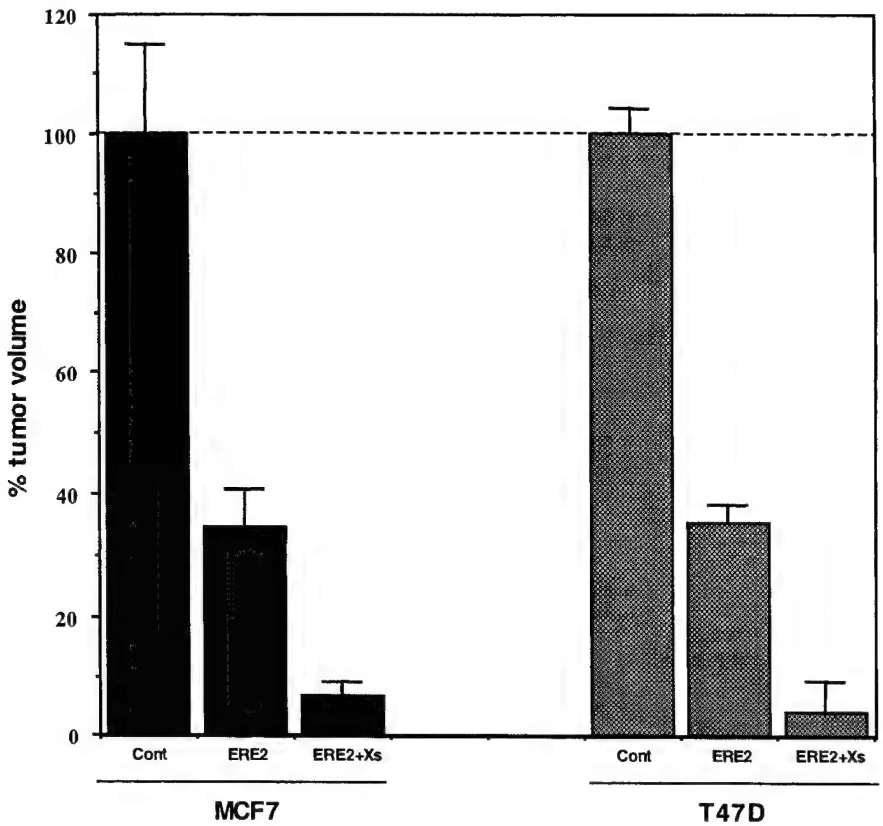


FIG. 10. Antitumoral effect of Ad5ERE2 in combination with Ad5Bcl-xs. Human tumor xenografts were established in nude mice by injection of MCF7 or T47D cells as described in Materials and Methods. In the case of MCF7, the tumors were randomized in three different groups ($n = 3$), which received one weekly intratumoral injection of vehicle alone (Cont), 10^7 PFU of Ad5ERE2 (ERE2), or 10^7 PFU of Ad5ERE2 plus 10^8 PFU of Ad5Bclxs (ERE2 + Xs). The T47D tumors received the same treatments except that the Ad5ERE2 dose was 5×10^7 PFU/tumor. Presented here is the average reduction of tumor volume 20 days after initiation of the treatment. The differences between each group were statistically significant ($p < 0.05$).

cells we show evidence that the virus preferentially kills ER^+ cells.

Conditionally replicative adenoviruses can act as a therapeutic tool themselves, but they can also deliver lethal genes to cancer cells to enhance their effect. In this way, the addition of a cassette for the expression of proapoptotic genes or prodrug-converting enzymes such as thymidine kinase or cytosine deaminase is technically possible (Wildner *et al.*, 1999). However, a conditionally replicative adenovirus can also be used to amplify the effect of any E1a-deleted adenoviral vector (Motoi *et al.*, 2000). We demonstrate that such viruses can be used to amplify a cytotoxic replication-deficient virus expressing the Bcl-xs gene, which is selectively toxic to transformed cells (Clarke *et al.*, 1995). The controlled replication of an adenovirus expressing such a gene can provide an additional level of specificity. The cooperation of Ad5ERE2 and Ad5Bcl-xs was demonstrated both *in vitro* and *in vivo*, using human tumor xenografts growing in nude mice.

In summary, the use of EREs to control the expression of E1a and E4 genes is a promising method for the construction of conditionally replicative viruses that can enhance cancer gene therapy strategies. It combines in the same therapeutic agent the targeting properties of the tissue-specific promoters with the exogenous modulation of the drug-regulated gene expression systems. We are currently using this approach to investigate the use of new hybrid promoters with improved tissue specificity as well as tumor-specific elements such as the hypoxia response elements.

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Advances in Brief

Overexpression of Bcl-x_S Sensitizes MCF-7 Cells to Chemotherapy-induced Apoptosis[1]

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Abstract

Resistance to apoptosis plays an important role in tumors that are refractory to chemotherapy. We report that Bcl-x_L, which functions like Bcl-2 to inhibit apoptosis, is highly expressed in MCF-7 human breast carcinoma cells. We used Bcl-x_S, a dominant negative inhibitor of Bcl-2 and Bcl-x_L, to demonstrate the role of these genes in modulating chemotherapy-induced apoptosis. Bcl-x_S overexpressed in MCF-7 cells by stable transfection does not affect viability by itself but induces a marked increase in chemosensitivity to VP-16 or taxol. Using an ELISA assay which quantitates DNA damage, we demonstrate that this sensitization is due to apoptosis, suggesting the therapeutic utility of targeting this pathway.

Introduction

An understanding of the molecular events responsible for the development of breast cancer may identify novel targets for therapeutic intervention. There is evidence that several chemotherapy agents used to treat cancers act through induction of apoptosis. The *p53* and *bcl-2* genes regulate apoptosis (1–4) and have been shown to directly affect the sensitivity of cancer cells to these agents. The Bcl-2 protein was first described as being overexpressed in follicular lymphomas, where it participates in tumor formation by blocking cell death (5, 6). Bcl-2 may play a role in breast cancer development since this gene is overexpressed in up to 70% of breast cancers (7). *In vitro* studies show that overexpression of Bcl-2 results in resistance to cisplatin and etoposide/VP-16-induced cytotoxicity, suggesting that Bcl-2 can inhibit apoptosis mediated by chemotherapy (3, 4). In other systems, Bcl-2 has been shown to block both p53-dependent and p53-independent cell death pathways (2–4). These studies suggest that strategies designed to block Bcl-2 might prove useful in sensitizing tumor cells to chemotherapy-induced apoptosis.

bcl-2 belongs to a family of genes involved in modulating apoptosis. The *bcl-x* gene is transcribed into two forms by alternate splicing (8). The product of the long form, Bcl-x_L, like Bcl-2, functions as an inhibitor of apoptosis (8). In contrast, the short form, Bcl-x_S, serves as a dominant negative inhibitor of both Bcl-x_L and Bcl-2 (8). We postulated that overexpression of Bcl-x_S in cells that express high levels of Bcl-2 or bcl-x_L would cause sensitization to chemotherapy-induced apoptosis. We report that Bcl-x_S-overexpressing MCF-7 cells are 5–10-fold more sensitive to apoptosis induced by the chemotherapy agents VP-16 or taxol than are MCF-7-*neo*-transfected control cells treated with these agents.

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Materials and Methods

Transfections. The MCF-7 human breast carcinoma cells were stably transfected with an expression plasmid encoding *bcl-x_S* (*pSFFVneo-bcl-x_S*) or a control plasmid (*pSFFVneo*; SFFV, spleen focus-forming virus). Transfections were performed using lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's protocol. The *bcl-x_S* or *neo*-transfected cells were subcultured in selection medium [containing MEM, 10% FCS, insulin (10 mg/liter or 10 μg/ml), and 1 mg/ml geneticin/G418 (GIBCO)] for 4 weeks. The MCF-7 clone with the highest Bcl-x_S expression, as determined by Western analysis, was used. Alternately, transient overexpression of Bcl-x_S was achieved by infecting MCF-7 cells with a replication-deficient adenovirus vector carrying the *bcl-x_S* minigene.[3]

Western Blot Analysis. Protein samples were prepared and resolved by denaturing SDS-PAGE using standard methods. The proteins were transferred to nitrocellulose and Western blotted with a mouse monoclonal antibody specific to human Bcl-x (a kind gift of Dr. Craig B. Thompson, Howard Hughes Medical Institute and Department of Medicine, Molecular Genetics & Cell Biology, University of Chicago, Chicago, IL). The specificity of this antibody has been demonstrated (9). A goat anti-mouse antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (Amersham, Arlington Heights, IL) were used to visualize protein bands.

Growth Studies. MCF-7-*neo* and *bcl-x_S* transfectants were cultured in medium lacking G418. Cells were plated in 12-well plates (10^4/well) and treated with/without varying doses of VP-16 (Sigma Chemical Co., St. Louis, MO) dissolved in DMSO (Sigma). Final DMSO concentrations (0.01–0.1%) were included in controls. Cells were treated with taxol (Paclitaxel; Bristol Myers Squibb Corp., Princeton, NJ) or cremophor (the carrier for taxol; Sigma). Cell viability was measured by hemocytometry using trypan blue exclusion.

Quantitation of Apoptosis by ELISA. To provide quantitative evidence in support of an apoptotic cell death mechanism, we used a "cell death" ELISA (Boehringer-Mannheim, Indianapolis, IN) that measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation. An anti-histone first antibody is coated on wells, which are then loaded with the cytoplasmic fraction of lysates from 2–5 × 10^3 cells/well. Cytoplasmic extracts from control and drug-treated cells were equalized on the basis of total cell number. The second antibody is an anti-DNA antibody conjugated to peroxidase. The ELISA was developed with peroxidase substrate, and the absorbance at $_{405}$ nM was measured using a Microplate autoreader (EL311; Bio-tek Instruments, Winooski, VT).

This ELISA measures cytoplasmic, DNA-bound histone as opposed to free histone or free DNA that may be non-specifically released into the cytoplasm during nonapoptotic cell death (10). As controls, cytoplasmic extracts from HL-60 cells treated with/without the topoisomerase I inhibitor, camptothecin, were used. Camptothecin-treated HL-60 cells demonstrated a DNA ladder (180 bp) when compared with untreated HL-60 cells (data not shown).

Statistical Analysis. Statistical significance was measured by Student's paired *t* test. *P* for each data set is shown in each figure legend.

[3] M. F. Clarke, I. Apel, V. N. Sumantran, J. J. Ryan, M. Gonzalez-Garcia, M. Benedict, N. Fukunaga, P. G. Eipers, B. Davidson, M. S. Wicha, and G. Nuñez. Activation of apoptosis in cancer cells by a *bcl-x_s* adenovirus vector, submitted for publication.

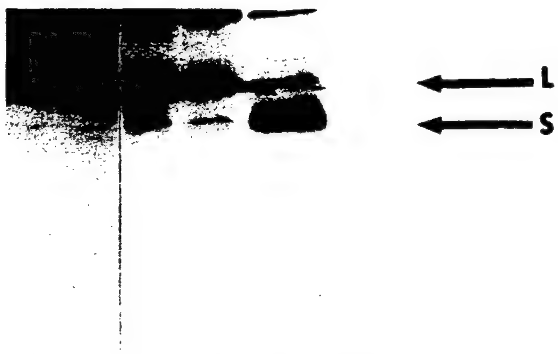

Fig. 1. Western blot of Bcl-x_L and Bcl-x_S expression. Total protein (80 μg) from each sample was loaded on 15% SDS-polyacrylamide gels. A mouse mAb to human Bcl-x was used to detect *Bcl-x_S*, which migrates at M_r 18,700 (*arrow S*), and *Bcl-x_L*, which migrates at M_r 25,630 (*arrow L*), proteins. The samples are: MCF-7-*neo* transfectants (duplicate samples of a cloned cell line; *Lanes 1* and 2) and MCF-7-*bcl-x_S* transfectants (duplicate samples of a cloned cell line; *Lanes 3* and *4*). The positive and negative controls: MCF-7 cells infected with an adenovirus vector expressing Bcl-x_S (*Lane 5*) or *lac Z* (*Lane 6*).

Results

Overexpression of Bcl-x_S in MCF-7 Cells. The expression of Bcl-x_L and Bcl-xS in MCF-7-*neo*- and *bcl-x_S*-transfected cells was assessed by Western blotting using a bcl-x monoclonal antibody specific for bcl-x proteins. As shown in Fig. 1, MCF-7-*neo*-transfected cells express large amounts, approximately 25-fold greater levels of Bcl-x_L compared to Bcl-x_S. Stable transfection of *bcl-x_S* into these cells results in approximately a 3.0-fold increased expression of Bcl-x_S compared to MCF-7-*neo*-transfected cells (Fig. 1, *Lanes 3* and *4 versus Lanes 1* and *2*). As positive and negative controls, protein samples were prepared from MCF-7 cells infected with an adenovirus vector expressing the *bcl-x_S* gene (Fig. 1, *Lane 5*) or the bacterial gene *lac Z* (Fig. 1, *Lane 6*).

Effect of Bcl-x_S Overexpression on Cell Viability. MCF-7 cells transfected with *bcl-x_S* showed approximately a 20% (± 6%; $n = 4$) decrease in growth rate compared to MCF-7-*neo* transfectants. However, in the absence of chemotherapy, the *bcl-x_S* transfectants maintained viability and did not undergo apoptosis (see below).

Effect of Bcl-x_S Overexpression on the Chemosensitivity of MCF-7 Cells to VP-16 and Taxol. We examined the sensitivity of MCF-7-*neo*- and *bcl-x_S*-transfected cells to the cytotoxic effects of VP-16. At concentrations of VP-16 from 0.5–2 μM, Bcl-x_S-overexpressing cells showed markedly increased cytopathic effects compared to MCF-7-*neo*-transfected cells treated with equivalent concentrations of VP-16 (Fig. 2). The most striking decrease in viable cell number in cultures of Bcl-x_S-overxpressing cells relative to MCF-7-*neo* control cells was observed at 2 μM VP-16 (Fig. 2, *D* and *H*). When the viability of cells shown in Fig. 2 was quantitated using trypan blue exclusion, we found that Bcl-x_S-overexpressing cells showed a 4.0- and 9.0-fold decrease in viability at 1 and 2 μM VP-16, respectively, compared to MCF-7-*neo* transfectants (Fig. 3*A*). As an additional method of measuring viability of VP-16-treated cells, we measured the replating efficiency of MCF-7-*neo versus* Bcl-x_S-overexpressing MCF-7 cells after VP-16 treatment.

Fig. 3*B* demonstrates that after VP-16 (2 μM) treatment for 6 days, Bcl-x_S-overexpressing MCF-7 cells showed a 2.5-fold decrease in replating efficiency when compared with VP-16-treated, MCF-7-*neo*-transfected cells. The untreated MCF-7-*neo* and *bcl-x_S* transfectants formed similar numbers of colonies after 6 days of treatment with DMSO alone (VP-16 solvent).

These studies indicate that as determined by morphology, replating efficiency, and viability (trypan blue exclusion), overexpression of Bcl-x_S sensitizes MCF-7 cells to cell death induced by low doses of VP-16.

Since overexpression of Bcl-x_S increased the sensitivity of MCF-7 cells to VP-16-induced death, it was important to determine if Bcl-x_S overexpression sensitized these cells to the apoptotic effects of other chemotherapy agents with different mechanisms of cytotoxicity. Taxol, whose mechanism of action differs from VP-16, has been shown to induce apoptosis less effectively in Bcl-2-overexpressing cells (11). Therefore, we measured viability of MCF-7-*neo* transfectants *versus* Bcl-x_S-overexpressing MCF-7 cells in the presence/absence of taxol (0.10 μM for 2 days). Fig. 3*C* shows that taxol caused a 15% decrease in the viability of the *neo* transfectants and a 60% decrease in the viability of Bcl-x_S-overexpressing MCF-7 cells. Thus, Bcl-x_S overexpression increased the chemosensitivity of MCF-7 cells to taxol and VP-16 by 4- and 9-fold, respectively.

As an alternate method of increasing Bcl-x_S expression, MCF-7 cells were infected with the adenovirus *bcl-x_S* or *lacZ* vectors (2000 particle-forming units/cell) and then treated with taxol (0.01 μM for 6 days). Viability studies showed that the *bcl-x_S*-infected cells were 3.25 ± 0.30-fold more sensitive to taxol-induced cell death than were uninfected cells or *lacZ*-infected cells ($n = 3$).

Effect of Bcl-x_S Overexpression on VP-16- and Taxol-induced Apoptosis. In mammary epithelial cells, demonstration of apoptosis has been difficult, since classical apoptotic morphology and DNA laddering have not been detected in cells undergoing death (12, 13). In order to provide evidence in support of an apoptotic cell death mechanism, we used a quantitative ELISA that measures cytoplasmic DNA histone complexes generated during apoptotic DNA fragmentation.

As measured by ELISA, MCF-7-*bcl-x_S* transfectants showed a 13- and 5-fold increase in apoptosis at 0.50 and 2 μM VP-16, respectively,

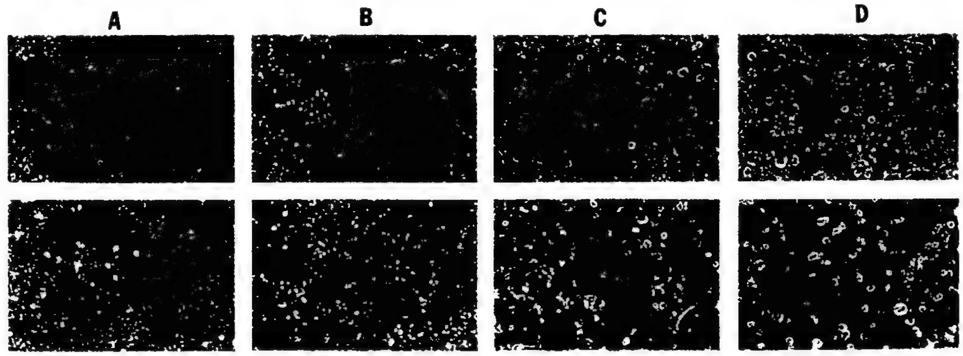

Fig. 2. Effect of VP-16 on MCF-7 cell morphology. MCF-7-*neo* (control) and *bcl-x_S* transfectants were treated with the indicated concentrations of VP-16 for 6 days and photographed at × 100. *A-D*, MCF-7-*neo*-transfected cells treated with 0, 0.5, 1.0, and 2.0 μM VP-16, respectively. *E-H*, MCF-7-*bcl-x_S* transfectants treated with 0, 0.5, 1, and 2 μM VP-16, respectively ($n = 6$).

when compared to *neo* transfectants treated similarly (Table 1A). Due to a high percentage of dead cells and fragile live cells in *bcl-x*$_S$ transfectants treated with 2 μM VP-16, some cell loss may have occurred during preparation of the ELISA sample. This may account for the decreased level of apoptosis measured in this sample compared to the sample from *bcl-x*$_S$ transfectants treated with 0.50 μM VP-16. Table 1A also shows that taxol induced a 5-fold higher level of apoptosis in MCF-7-*bcl-x*$_S$ transfectants than in MCF-7-*neo* control cells. Thus, the ELISA measured 5–10-fold increased cytoplasmic DNA in VP-16- or taxol-treated MCF-7-*bcl-x*$_S$ transfectants compared to MCF-7-*neo*-transfected cells treated similarly.

Taxol-induced cell death in MCF-7 cells infected with the *bcl-x*$_S$ or *lacZ* adenoviral vectors was measured by ELISA. We observed a significant level of apoptosis in *bcl-x*$_S$-infected cells relative to uninfected or lacZ-infected cells in the absence of taxol, suggesting that transient overexpression of Bcl-x$_S$ itself can induce apoptosis (Table 1B). However, taxol induced a 24-fold increase in apoptosis in *bcl-x*$_S$-infected cells compared to a 5-fold higher level of apoptosis in the uninfected or lacZ-infected cells treated with the same dose of taxol (Table 1B). Thus, transient overexpression of Bcl-x$_S$ sensitizes MCF-7 cells to taxol-induced apoptosis, a result that is consistent with that observed in MCF-7 cells stably transfected with *bcl-x*$_S$ (Table 1).

These results are indicative of increased DNA fragmentation in Bcl-x$_S$-overexpressing MCF-7 cells undergoing VP-16- or taxol-induced apoptosis, suggesting that the increased chemosensitivity of these cells to VP-16 or taxol is in part due to a marked increase in chemotherapy-induced apoptosis.

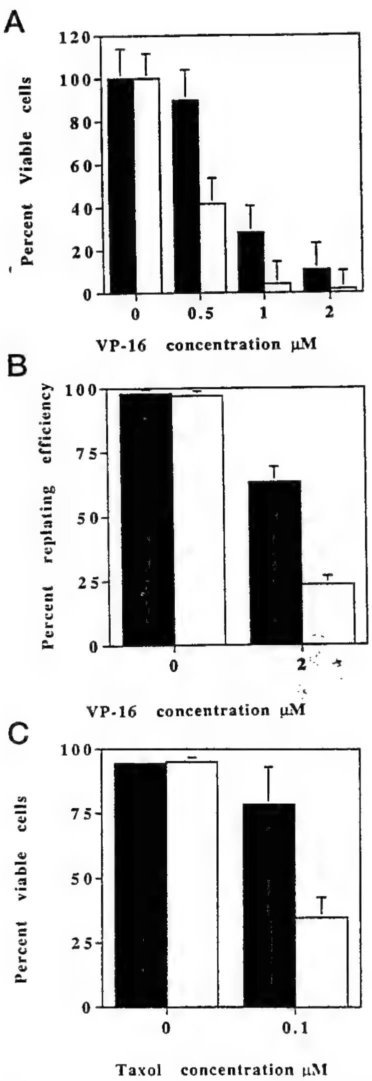

Fig. 3. Effect of VP-16 and taxol on viability of Bcl-x$_S$-overexpressing MCF-7 cells. MCF-7-*bcl-x*$_S$ (□)- or *neo* (■)-transfected cells were treated with or without the indicated concentrations of VP-16 or taxol. *A*, percentage viability of both cell lines treated with/without VP-16 (0–2.0 μM) for 6 days (*bars*, SE; $n = 3$; $P \leq 0.01$). *B*, percentage replating efficiency of VP-16-treated cells. MCF-7-*bcl-x*$_S$-transfected cells (□) and MCF-7-*neo* cells (■) were treated with/without VP-16 (2 μM for 6 days). On day 6, cells from each treatment were harvested and replated at a density of 1000 or 2000 cells/well in triplicate wells. After 10 days, colonies that formed from each sample were counted. Replating efficiency of VP-16-treated *bcl-x*$_S$ cells is expressed as the percentage of colonies formed relative to that of the untreated *bcl-x*$_S$ transfectants. Similarly, the replating efficiency of VP-16-treated MCF-7-*neo* transfectants is expressed relative to that of untreated MCF-7-*neo*-transfected cells (*bars*, SE; $n = 3$; $P \leq 0.001$). *C*, the percentage viability of MCF-7-*bcl-x*$_S$ (□) and MCF-7-*neo* cells (■) treated with/without 0.10 μM taxol for 2 days (*bars*, SE; $n = 3$; $P \leq 0.01$).

Table 1A *Quantitative ELISA for DNA fragmentation of stably transfected cells after VP-16 or taxol treatment*

MCF-7-*bcl-x*$_S$ and *neo* transfectants were treated with or without the indicated concentrations of VP-16 for 6 days or taxol for 2 days. Cytoplasmic extracts were prepared from an equal number of total (live + dead) cells from each sample for ELISA. These extracts were assayed for cytoplasmic DNA-histone with the use of a cell death ELISA ($n = 3$; $P \leq 0.10$ for VP-16-treated samples and $P \leq 0.001$ for taxol samples).

	Fold increase in apoptosis[a] Mean units of cytoplasmic DNA-histone (± SEM)[b]			
Sample	Control	0.5 μM VP-16	2 μM VP-16	0.10 μM taxol
MCF-7 *neo*	1.00	2.22 (0.62)	6.68 (1.11)	2.67 (1.09)
MCF-7 *bcl-x*$_S$	1.00	29.05 (9.00)	37.32 (9.96)	13.65 (0.95)

Table 1B *Quantitative ELISA for DNA fragmentation in adenovirus-infected cells after taxol treatment*

MCF-7 cells were infected with the *bcl-x*$_S$ or *lacZ* adenoviral vectors (2000 particle-forming units/cell). Cells were treated with or without taxol for 6 days. ELISA samples were prepared as described (see Table 1A) ($n = 3$; $P \leq 0.01$).

	Mean units of cytoplasmic DNA-histone (± SEM)[b]		
Sample	Uninfected	*bcl-x*$_S$ infected	*lac-Z* infected
Control	1.00	6.07 (1.94)	1.32 (0.25)
Taxol (0.01 μM)	5.01 (1.30)	23.80 (0.60)	5.68 (2.35)

[a] Fold increase in apoptosis = units of DNA-histone in treated cells/units of DNA-histone in untreated cells.

[b] 1.0 units$_{405}$ nM = peroxidase activity conjugated to anti-DNA-anti-histone specifically bound to a cytoplasmic extract from 2×10^3 HL-60 cells treated with camptothecin (2 μg/ml for 4 h). The basal levels of cytoplasmic DNA-histone in untreated MCF-7-*neo* and *bcl-x*$_S$-transfected cells ranged from 0.05 to 0.150 units and were normalized to a value of 1.0 (**Table 1A**). Basal levels of cytoplasmic DNA-histone in untreated uninfected MCF-7 cells ranged from 0.02 to 0.10 units and were normalized to a value of 1.0 (**Table 1B**).

Discussion

To examine the role of the *bcl-2* family of genes in mediating chemotherapy-induced apoptosis in breast cancer, we used MCF-7 human mammary carcinoma cells. These cells have been reported to express wild-type p53 (14) and high levels of Bcl-2 (15). In addition, we found that these cells express high levels of Bclx-$_L$ compared to Bcl-x$_S$ (Fig. 1).

The finding that MCF-7 cells express predominantly inhibitors of apoptosis, Bcl-2 and Bcl-x$_L$ allowed us to determine the effect of stable transfection of the dominant negative inhibitor of this pathway, Bcl-x$_S$, on apoptosis in these cells. Stable transfection of *bcl-x*$_S$ resulted in approximately a 3-fold increase in Bcl-x$_S$ expression in MCF-7 cells compared to *neo* transfectants. However, the *bcl-x*$_S$ transfectants still expressed approximately 8-fold greater levels of Bcl-x$_L$ compared to Bcl-x$_S$ (Fig. 1). The inability to produce stable transfectants that expressed higher levels of Bcl-x$_S$ suggested that high levels of this protein might prove lethal. Indeed, we have found that higher levels of Bcl-x$_S$ expression produced by *bcl-x*$_S$ adenovirus

infection of MCF-7 cells compared to stable transfection with plasmid-encoded *bcl-x_S* decreased cell viability, even in the absence of chemotherapy. Furthermore, the increased levels of Bcl-x_S expression obtained by infection with the *bcl-x_S* adenoviral vector sensitized these cells to taxol-induced apoptosis (Table 1B). This observation suggests that the effects of overexpression of Bcl-x_S on chemosensitivity in the stably transfected cells are not simply an artifact of transfection or clonal selection.

In this report, we demonstrate that overexpression of Bcl-x_S in MCF-7 cells sensitizes these cells to apoptosis induced by low concentrations of VP-16 or taxol. Both of these agents have clinical utility in treating breast cancer (16, 17). VP-16, a topoisomerase II inhibitor, induces DNA damage and subsequent apoptosis. Taxol is cytotoxic because it inhibits mitotic spindle function. The observation that overexpression of Bcl-x_S causes sensitization to apoptosis by two chemotherapy agents with different mechanisms of action suggests that these agents induce cell death by a common pathway inhibited by Bcl-2 and/or Bcl-x_L.

Recently, proteins that modulate apoptosis have been described. BAG-1 is a Bcl-2 binding protein that inhibits apoptosis (18), whereas the Bad and Bax proteins promote apoptosis (19, 20). Thus, the apoptotic threshold of a cell may be the result of a dynamic balance between the positive and negative regulators of apoptosis.

The molecular mechanism by which Bcl-x_S overexpression increases chemosensitivity of MCF-7 cells remains to be determined. Interactions between Bcl-2, Bclx-$_L$,and Bcl-x_S proteins have been analyzed using the yeast two-hybrid system. These studies suggest that the Bcl-xS protein can heterodimerize with Bcl-2 and Bcl-x_L (21). Thus, loss of Bcl-x_L and/or Bcl-2 function upon Bcl-x_S binding is a plausible mechanism by which Bcl-x_S-overexpressing MCF-7 cells become sensitized to chemotherapy-induced apoptosis. The relative overabundance of Bcl-x_L over Bcl-x_S, even in the stably transfected cells, suggests that other factors may be involved. For example, the Bax and Bad proteins may also play important roles in modulating the chemosensitivity of MCF-7 cells.

Our demonstration that overexpression of Bcl-x_S in MCF-7 human breast carcinoma cells induces sensitization to apoptosis induced by low concentrations of VP-16 and taxol provides evidence that Bcl-x_L and Bcl-2 play a role in modulating apoptosis in these cells. Furthermore, these studies suggest that development of pharmacological agents that target this pathway represents a rational therapeutic strategy.

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MANUSCRIPT #2
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Medical Sciences

A recombinant *bcl-x_S* adenovirus selectively induces apoptosis in cancer cells but not in normal bone marrow cells

(*bcl-2*/gene therapy/stem cells)

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ABSTRACT Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x_S*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating severe combined immunodeficient mice were refractory to killing by the *bcl-x_S* adenovirus. These results suggest that Bcl-2 family members are required for survival of cancer cells derived from solid tissues. The *bcl-x_S* adenovirus vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation.

It is becoming increasingly apparent that disruption of the pathways regulating programmed cell death (PCD; apoptosis) is integral to the etiology of a variety of cancers. Expression of certain tumor-suppressor proteins such as p53 can induce some cancer cells to undergo apoptosis (1–4). Oncogenes have also been implicated in PCD. For example, several groups have observed that deregulated expression of c-*myc* can activate the apoptosis pathway (5–7). Furthermore, *bcl-2*, the gene deregulated in most follicular lymphomas, primarily functions to inhibit apoptosis (for reviews, see refs. 8 and 9). *bcl-2* encodes an intracellular membrane-associated protein that has been localized to the mitochondria, endoplasmic reticulum, and perinuclear regions (9). Although expression of *bcl-2* does not stimulate cell proliferation, it can cooperate with c-*myc* (10, 11) to cause transformation. Moreover, expression of high levels of Bcl-2 protein in normal or neoplastic cells delays or inhibits PCD induced by many factors including p53, Myc, chemotherapy, and ionizing radiation (8, 9). A large percentage of epithelial and hematopoietic tumors overexpress Bcl-2 (8, 9). Furthermore, overexpression of Bcl-2 is correlated with poor prognosis and resistance to treatment in patients with neuroblastoma (12), prostatic cancer (13), and some forms of leukemia (14).

Recently, a homolog of *bcl-2*, called *bcl-x*, has been identified and partially characterized (15, 16). As a result of alternative splicing, two *bcl-x* mRNA species, designated *bcl-x_L* and *bcl-x_S*, were identified in the human. The former, like *bcl-2*, inhibits apoptosis (15, 17). The latter is thought to function as a repressor of Bcl-2, as it enhances apoptotic signals in cells that express Bcl-2 (15). Postulating that inactivation of Bcl-2 or Bcl-x_L might increase the susceptibility of cancer cells to PCD, an adenovirus vector that expresses Bcl-x_S protein was constructed. Primary carcinoma cells, as well as cell lines derived from solid tumors, rapidly underwent cell death after infection with the *bcl-x_S* adenovirus. In primary breast cancer cells and multiple breast cancer cell lines, expression of *bcl-x_S* was associated with rapid induction of cell death. In contrast, human hematopoietic progenitor cells exposed to this virus maintained viability and retained their ability to reconstitute the bone marrow of irradiated immune-deficient mice. Blocking Bcl-2 or Bcl-x_L function by the *bcl-x_S* adenovirus appears to provide another strategy for inducing apoptosis in tumor cells. These findings have important implications for cancer therapy.

MATERIALS AND METHODS

Construction of the *bcl-x_S* Adenovirus. The plasmid pBSbcl-x_S (15) was digested with *Eco*RI, and customized *Bam*HI linkers were ligated onto the ends; the ≈536-bp fragment was purified and ligated into *Bam*HI-digested pAd5RSV plasmid (18). The pAd5RSV *bcl-x_S* recombinant virus was isolated by *in vivo* homologous recombination between the linearized pAd5RSV *bcl-x_S* plasmid and the replication-deficient sub360 adenovirus that has a partial deletion of the E3 region and deletion of the E1A and E1B genes (18). Three recombinant viruses that expressed Bcl-x_S protein in infected cells were plaque-purified twice. Large preparations of adenovirus were made by infecting 293 cells and purifying crude virus preparations by CsCl centrifugation (19).

Cell Infections with Recombinant Adenoviruses. The number of adenovirus particles in viral stocks was determined by spectrophotometry (18). Adenovirus titers were determined by limiting dilution and plaque formation of 293 cells exposed to the virus dilutions. Absence of replication-competent virus was confirmed by limiting dilution and plaque formation of HeLa cells exposed to the virus dilutions. Each cell line was infected with a stock of the β-galactosidase virus of known titer and then stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) to determine the number of viruses per cell needed to infect 85–98% of each cell line. Unless otherwise indicated, the concentration of the *bcl-x_S* adenovirus used to infect cells was identical to the number of β-galactosidase viruses that infected 85–98% of the cells. Cells were exposed to the adenovirus vectors for 4 hr in serum-free medium. The medium was then replaced with tissue culture medium/2% fetal calf serum, and the cells were incubated overnight. The next day the medium was removed and replaced with tissue culture medium/10% serum. Cell viability was measured by trypan blue exclusion.

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Abbreviations: PCD, programmed cell death; SCID, severe combined immunodeficiency virus.

Analysis of Bcl-x Protein. Immunoblot analysis of Bcl-x proteins was done as described (17) by using a rabbit anti-Bcl-x antiserum. The blots were developed with epichemiluminescence substrate (Amersham).

Hematopoietic Cell Assays. Bone marrow was harvested from normal human volunteers by using a protocol approved by the University of Michigan Institutional Review Board. Low-density mononuclear cells were isolated using Ficoll/Hypaque centrifugation essentially as described (20). Hematopoietic cells (1×10^6) were mixed with 1.5×10^4 pSV$_2$-neo-transfected SHSY-5 neuroblastoma cells. The cells were then infected with adenovirus in serum-free medium containing kit ligand at 1 mg/ml and interleukin 3 at 10 mg/ml. After 2 days of culture, cells were harvested, and triplicate progenitor assays using 1×10^4 cells were done as described (20). To assay for viability of SHSY-5 cells that had been mixed with hematopoietic cells and then exposed to the *bcl-x$_S$* adenovirus, cells were grown in tissue culture medium containing the antibiotic Geneticin at 1 mg/ml to kill the normal hematopoietic cells. To determine the ability of *bcl-x$_S$* adenovirus-infected cells to engraft nonobese diabetic (Nod)/severe combined immunodeficiency (SCID) mice, 1×10^7 low-density bone marrow mononuclear cells were infected with 0, 2×10^3, 5×10^3, or 10^4 adenoviruses per cell and inoculated into the tail vein of irradiated (400 cGy) Nod/SCID mice (21). After 1 mo, the mice were sacrificed, and the bone marrow was harvested and analyzed for human hematopoietic cells essentially as described (22).

RESULTS

Construction of Adenoviral Vector Expressing Bcl-x$_S$. We and others have recently demonstrated that the overexpression of Bcl-2 oncogene will block p53-induced apoptosis (9). This finding led to the prediction that inhibition of Bcl-2 function might induce apoptosis in tumor cells that express wild-type p53. To test this hypothesis, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of Bcl-2, was constructed by inserting the *bcl-x$_S$* coding sequences into the pADRSV vector (Fig. 1*A*). Nine virus plaques were isolated by cotransfecting the pADRSV *bcl-x$_S$* construct with the sub360 adenovirus into 293 human kidney cells (23), and the viruses were amplified. Restriction digests and Southern blots revealed three viruses that contained the *bcl-x$_S$* minigene. Immunoblotting using a rabbit polyclonal antibody raised against the Bcl-x protein revealed that MCF-7 breast cancer cells infected with the *bcl-x$_S$* adenovirus, but not with a control adenovirus that contains a β-galactosidase gene, expressed the ≈21-kDa Bcl-x$_S$ protein (Fig. 1*B*).

The *bcl-x$_S$* Adenovirus Is Lethal to a Broad Range of Cancer Cells. MCF-7 breast cancer cells (which express high levels of wtp53 and Bcl-2) infected with the *bcl-x$_S$* adenovirus, but not MCF-7 cells infected with the control virus, began dying 2 days after infection. When cells were analyzed 6 days after infection, the MCF-7 cells infected with the β-galactosidase adenovirus had grown and formed colonies. In contrast, MCF-7 cells infected with the *bcl-x$_S$* adenovirus became rounded, subsequently detached from the tissue culture plastic, and died (Fig. 2*A*). Dying cells infected with the *bcl-x$_S$* adenovirus morphologically resembled cells undergoing apoptosis. They were shrunken with picnotic nuclei and cytoplasmic blebbing (Fig. 2*A*). Furthermore, DNA degradation, a hallmark of apoptosis, was detected *in situ* in virtually all cancer cells infected with the *bcl-x$_S$* adenovirus but not with the control virus using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay (24) (data not shown). Several other types of cancer cells including cells of breast, colon, and neuroblastoma origin were also killed by the *bcl-x$_S$* adenovirus (Table 1). As reported, the adenovirus containing the β-galactosidase gene alone demonstrated various degrees of toxicity to some but not all cancer cell lines (25). To determine whether the *bcl-x$_S$* adenovirus can induce cell death in primary cancer cells, breast cancer cells isolated from six patients were exposed to the virus. When infected with β-galactosidase virus at 1×10^3 to 1×10^4 viruses per cell, there was no effect on viability (Fig. 2*B*). In contrast, cells infected with even the lowest concentration of the *bcl-x$_S$* adenovirus showed a marked cytotoxicity (Fig. 2*B*). Primary cells isolated from one of the patients would form colonies in tissue culture. Fifty thousand cells from this patient were cultured after infection with zero or 1×10^4 viruses per cell. Although innumerable colonies formed in the control cultures, none formed in the cells infected with the *bcl-x$_S$* adenovirus (data not shown). The *bcl-x$_S$* adenovirus was also lethal to primary breast cancer cells isolated from five other patients (Table 1).

The ability of *bcl-x$_S$* adenovirus-infected cells to grow *in vivo* was tested. Two days after 5×10^4 RKO colon cancer cell were infected with the *bcl-x$_S$* adenovirus, but not the β-galactosidase virus, the cells began dying. By day 6, only a few of th *bcl-x$_S$* adenovirus-infected cells excluded trypan blue (data nc shown). Next, 5 million RKO cells were infected with 2×10 *bcl-x$_S$* adenoviruses per cell or β-galactosidase viruses per cel Uninfected cells or colon cancer cells infected with the contr virus formed tumors in 7 out of 10 and 2 out of 5 injected nuc mice, respectively (Table 2). In contrast, RKO cells infecte with the *bcl-x$_S$* adenovirus did not form tumors in any of tl 15 mice injected with such cells (Table 2).

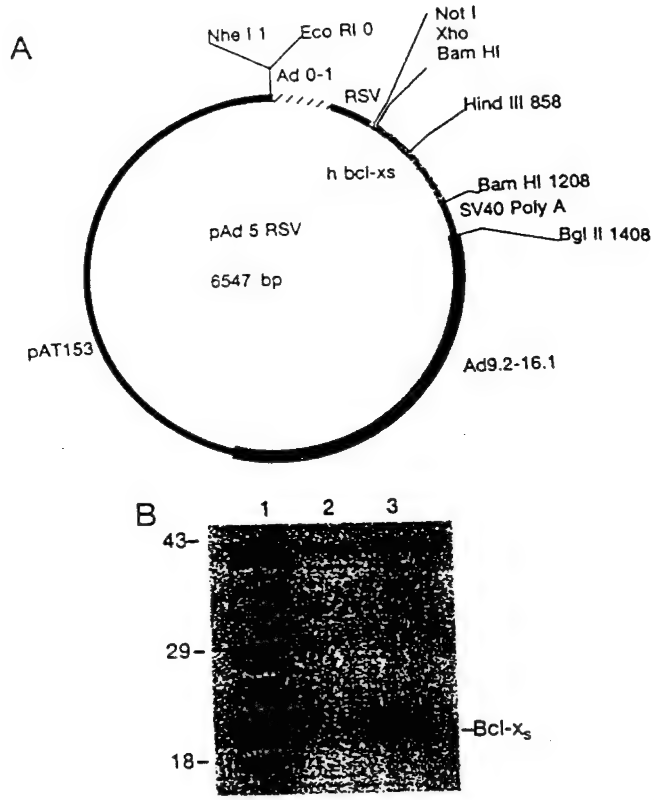

FIG. 1. (*A*) The *bcl-x$_S$* adenovirus. The pRSVAd/*bcl-x$_S$* construct is shown. SV40, simian virus 40; Ad, adenoma virus. (*B*) Immunoblot of lysates from MCF-7 cells. Expression of Bcl-xs protein was analyzed by SDS/PAGE and immunoblotting with a rabbit polyclonal antiserum (17). Lanes: 1, 200 μg of protein from FL5.12 cells transfected with pSFFV/*bcl-x$_S$* minigene (15); 2, 40 μg of protein from the parental MCF-7 cells infected with β-galactosidase adenovirus; 3, 40 μg of protein from MCF-7 cells infected with *bcl-x$_S$*-adenovirus. Molecular size standards are shown at left (in kDa). Note that MCF-7 cells infected with *bcl-x$_S$* adenovirus express the ≈21-kDa Bcl-xs protein.

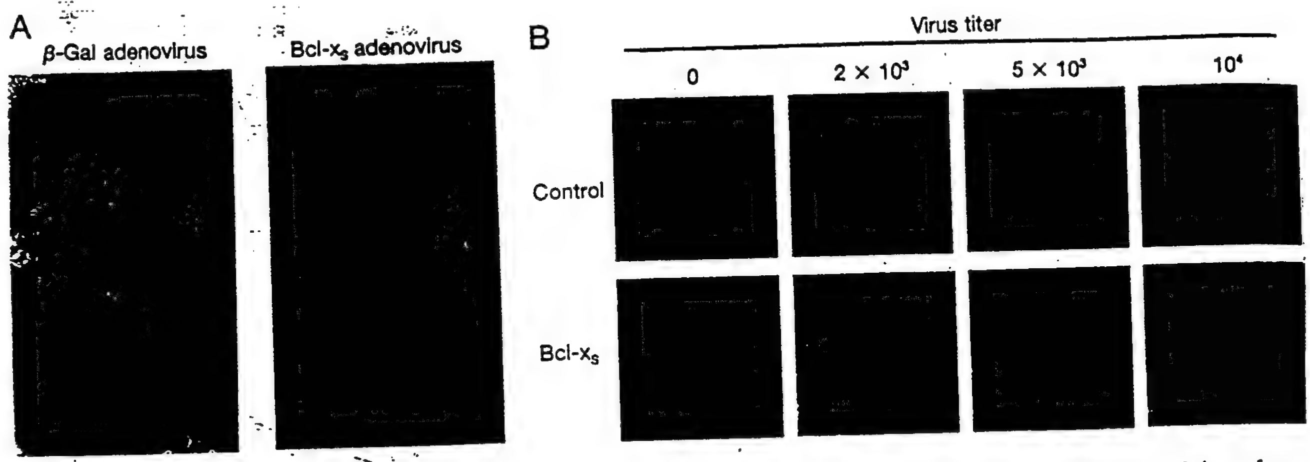

FIG. 2. (*A*) Microphotograph of MCF-7 cells. MCF-7 cells (5 × 10^5) infected with the indicated virus photographed after 6 days of growth. β-Gal, β-galactosidase. Note that virtually all cells infected with *bcl-xs* adenovirus have died. (×50.) (*B*) Photomicrograph of adenovirus-infected primary breast cancer cells. Photomicrographs were taken of primary breast cancer cells infected 2 days previously with the indicated titer of β-galactosidase adenovirus (control) or the *bcl-xs* adenovirus. Cells infected with even the lowest titer of *bcl-xs* adenovirus show evidence of viral toxicity, whereas cells infected with even the highest titer of β-galactosidase virus remained viable. (×270.)

bcl-xs Adenovirus Cytotoxicity Is Selective for Tumor Cells but Spares Human Hematopoietic Cells. High-dose chemotherapy followed by infusion of autologous bone marrow to rescue the damaged hematopoietic system is felt to cure some children with neuroblastoma (26). Unfortunately, the bone marrow of such patients is often contaminated with neuroblastoma cells that contribute to relapse (27). To mimic the situation in which bone marrow cells collected for bone marrow transplantation are contaminated with cancer cells, 1 × 10^6 low-density human bone marrow mononuclear cells were mixed with 1.5 × 10^4 SHSY-5 human neuroblastoma cells. The lowest virus concentration (2 × 10^3 viruses per cell) completely inhibited the ability of SHSY-5 neuroblastoma cells to form colonies (data not shown). After exposure of the bone marrow cells to 2–10 × 10^3 viruses per cell, which totally inhibited proliferation of the neuroblastoma cells, human hematopoietic progenitor cells remained viable and formed colonies in methylcellulose (Fig. 3). There was a slight decrease in hematopoietic cell colonies after exposure to 1 × 10^4 viruses per cell. This result was not specific for the *bcl-xs* adenovirus because it was observed with a control adenovirus (data not shown) and is probably secondary to nonspecific viral particle toxicity at very high doses (B.D., unpublished observation).

Table 1. Viability of different cancer cells after exposure to *bcl-xs* adenovirus

	Cytotoxicity	
Cells	β-Galactosidase adenovirus	*bcl-xs* adenovirus
RKO (human colon carcinoma)	−	++++
Primary breast carcinoma cells*		
Patient 1	−	++++
Patient 2	−	++++
Patient 3	+	++++
Patient 4	−	++++
Patient 5	+	++++
Patient 6	−	++++
MDA435 (human breast carcinoma)	+++	++++
T47D (human breast carcinoma)	+++	++++
MCF-7 (human breast carcinoma)	++	++++
HT29 (human colon carcinoma)	++	++++
SHSY-5 (human neuroblastoma)	+	++++
SK-N-SH (human neuroblastoma)	+	++++
IMR-32 (human neuroblastoma)	++++	++++
K-562 (human leukemia)	−	−

The indicated cell lines were infected with *bcl-xs* adenovirus by using a virus titer that resulted in expression of β-galactosidase in >95% of cells infected with the same titer of β-galactosidase virus. In cell lines, each experiment was done in triplicate, and viability was determined 6 days after infection. Degree of cytoxicity was as follows: − (<5%), + (6–25%), ++ (26–50%), +++ (51–90%), and ++++ (91–100%).
*Breast cancer cells isolated from either pleural or ascites fluid were collected by the University of Michigan tissue procurement laboratory and stored in liquid nitrogen. These cells were placed in tissue culture medium and exposed to the *bcl-xs* adenovirus. The primary breast cancer cells were infected with identical titers of either β-galactosidase or *bcl-xs* adenovirus that resulted in expression of β-galactosidase in most cells. The primary breast cancer cells infected with β-galactosidase virus remained viable.

Hematopoietic Cells Exposed to the *bcl-xs* Adenovirus Retained the Ability to Reconstitute Bone Marrow. For the *bcl-xs* adenovirus to be clinically effective in tumor cell purging, human hematopoietic stem cells capable of repopulating the patient must be spared. Recently, transplantation assays for primitive human SCID-repopulating cells (21) have been developed by engrafting human bone marrow or cord blood in irradiated immune-deficient SCID or Nod/SCID mice (22.

Table 2. Tumor formation in nude mice

Adenovirus	Mice injected, no.	Tumors, no.
Mock	10	7
β-Galactosidase	5	2
bcl-xs	15	0

RKO colon cancer cells were infected with 10^3 of the indicated adenovirus per cell. Approximately 16 hr later, 5 × 10^6 cells were collected and injected into the flanks of nude mice. Control cells (either mock-infected cells or β-galactosidase adenovirus-infected cells) were injected into one flank, and *bcl-xs* adenovirus-infected cells were injected into the opposite flank of 15 mice. Mice were examined 4 weeks later for tumors. Statistical analysis using the Wilcoxon signed-rank analysis shows a significant difference in the number of tumors that the control cells vs. the *bcl-xs* adenovirus-infected cells formed (P = 0.018).

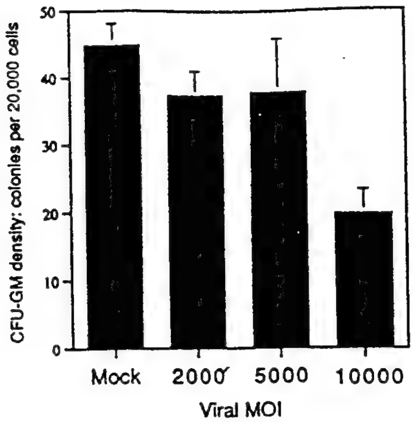

FIG. 3. Mononuclear cells from normal human bone marrow were isolated as described (20). Duplicate samples of hematopoietic cells were infected with the indicated number of *bcl-xs* adenoviruses per cell, and then progenitor assays were done in triplicate. Note that only at the highest virus concentration is there any decline in colony numbers. Data from one experiment are shown. A second experiment using a different donor yielded essentially identical results. MOI, multiplicity of infection.

28). To ensure that SCID-repopulating cells remained functional after exposure to the *bcl-x_S* adenovirus, treated human bone marrow cells were transplanted into Nod/SCID mice. Human bone marrow mononuclear cells were exposed to up to 1×10^5 *bcl-x_S* adenovirus vector per cell and cultured *in vitro.* The hematopoietic cells (1×10^7) were then infused into the tail vein of irradiated mice according to standard protocols (21, 22). One month after transplantation, DNA analysis with a human-specific α-satellite probe indicated that significant levels of human cells had repopulated the mouse bone marrow (Table 3). In addition, the bone marrow contained multiple lineages of human myeloid and erythroid progenitors, even in mice transplanted with cells exposed to the highest virus titer (Table 3). Quantitatively and qualitatively, these mice were indistinguishable from several hundred mice that we have transplanted with normal human cells, indicating that the SCID-repopulating cells were unaffected by exposure to the *bcl-x_S* adenovirus. These data suggest the feasibility of using the *bcl-x_S* adenovirus vector to eliminate cancer cells from the bone marrow while sparing normal stem cells.

DISCUSSION

It has been postulated that Bcl-2 may contribute to the malignant phenotype by blocking apoptotic pathways in cancer cells. In this present report, we used an adenovirus vector containing *bcl-x_S*, a functional inhibitor of Bcl-2, to induce PCD in human cancer cells derived from a variety of solid tumors. Furthermore, our evidence shows that the cytotoxicity induced by the *bcl-x_S* adenovirus vector is cell-type specific because normal human bone marrow hematopoietic progenitor cells are resistant to *bcl-x_S* adenovirus-induced apoptosis. Indeed, human hematopoietic cells exposed to these viruses retained the ability to reconstitute the bone marrow of irradiated SCID mice.

Initial descriptions of Bcl-x_S suggested that expression of this protein inhibited the ability of Bcl-2 to protect cells from PCD induced by interleukin 3 withdrawal (15). It is notable that the *bcl-x_S* adenovirus is uniformly lethal to all solid tumor cells thus far tested. In contrast, the *bcl-x_S* adenovirus failed to induce cell death in hematopoietic precursors. The mechanism for this cell-type specificity is presently unknown. Expression of *bcl-x* is obligate for fetal liver hematopoiesis (29). However, it is not known whether expression of Bcl-x_L is necessary for adult hematopoiesis or at what stage of differentiation expression of *bcl-x_L* is required for survival. The cell-type selectivity of the *bcl-x_S* adenovirus may, at least partly, be due to the fact that these recombinant adenoviruses do not result in prolonged expression of transgenes in hematopoietic stem cells and that expression of Bcl-x_L is not required for such cells to survive. It is also possible that the *bcl-x_S* adenovirus does not infect stem cells. Recent evidence shows that adenovirus vectors demonstrate tissue specificity. In lung tissue recombinant adenoviruses do not efficiently transduce columnar epithelial cells *in vivo* (30).

The observation that the *bcl-x_S* adenovirus is uniformly toxic to such a diverse number of cancer cells suggests that expression of a *bcl-2* family member may be obligate for cell survival in cancer cells of solid tissue origin. It has been thought that Bcl-2 and Bcl-x_L proteins protect cells from apoptotic signals such as those induced by growth factor withdrawal or DNA damage (8, 9). Our results suggest that such signals might be constitutively present in certain cells. Cells stably transfected with a *bcl-x_S* plasmid and grown using selection medium in culture (15) uniformly express a small amount of Bcl-x_S protein compared with cells infected with the *bcl-x_S* adenovirus (G.N., unpublished data). Thus, efficient induction of apoptosis by

Table 3. Human hematopoietic cell engraftment of SCID mice

	Human cells, %	Colonies: BFU-E	CFU-G	CFU-M	CFU-GM	CFU-GEMM	Total
Mock infection	1–10	3	27	18	0	1	49
Mock infection	0	1	4	0	0	0	5
2K virus infection	10–50	22	43	81	4	1	151
2K virus infection	10–50	38	77	157	4	2	278
5K virus infection	1–10	2	9	13	0	0	24
5K virus infection	N/A	—	—	—	—	—	—
10K virus infection	1–10	2	15	24	1	1	43
10K virus infection	1–10	2	17	16	2	0	37

Low-density mononuclear cells from human bone marrow were collected as described (20) and infected with the indicated number of the *bcl-x_S* adenoviruses per cell. The next day, irradiated SCID mice were injected with $\sim 1 \times 10^7$ cells essentially as described (21, 22). After 1 mo, bone marrow cells were harvested. Southern blots were done to determine the percentage of human cells in the bone marrow (22). Low-density mononuclear cells were cultured in duplicate in methylcellulose with human hematopoietic growth factors, and erythroid (BFU-E), granulocyte (CFU-G), macrophage (CFU-M), granulocyte/macrophage (CFU-GM), and mixed granulocyte/erythroid/monocyte (CFU-GEMM) colonies were counted 2 weeks later. One of the mice injected with cells that were exposed to 5×10^3 (5K) viruses per cell died before analysis. Note that mouse marrow was engrafted with human hematopoietic cells exposed to the highest titer of virus.

the *bcl-x*$_S$ adenovirus may relate to its ability to transduce high levels of Bcl-x$_S$ protein in infected cells. An alternative explanation is that cancer cells infected with the *bcl-x*$_S$ adenovirus express an effector of apoptosis, either an endogenous effector or a virally encoded effector (or both).

Cancer cell contamination of bone marrow used to rescue patients from high-dose chemotherapy is a significant problem in the treatment of neuroblastoma (31) and breast cancer (32). Elegant retrovirus-tagging experiments have shown that reinfusion of malignant cells contributes to the relapse of neuroblastoma (27). In all relapsed patients, biopsies of such tumors showed that virally marked cells were invariably present (27). We have shown that after infection of contaminated bone marrow cells with the *bcl-x*$_S$ adenovirus, the cells can be incubated *in vitro* for a short period to allow the carcinoma cells to die and then be infused into a mouse and reconstitute hematopoiesis. By this method, the *bcl-x*$_S$ adenovirus can be used as a "molecular scalpel," either by itself or in conjunction with other purging techniques (33), to selectively eliminate contaminating tumor cells from bone marrow samples. Together, these observations suggest that strategies such as the *bcl-x*$_S$ adenovirus designed to disrupt the *bcl-2* family pathway may provide alternative therapeutic approaches to cancer treatment.

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MANUSCRIPT #3
From manuscript and abstract list in references

[CANCER RESEARCH 56, 1965–1969, May 1, 1996]

Advances in Brief

bcl-x_s Gene Therapy Induces Apoptosis of Human Mammary Tumors in Nude Mice[1]

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Abstract

Bcl-x$_s$ is a dominant negative repressor of Bcl-2 and Bcl-x$_L$, both of which inhibit apoptosis. We used a replication-deficient adenoviral vector to transiently overexpress Bcl-x$_s$ in MCF-7 human breast cancer cells, which overexpress Bcl-x$_L$. Infection with this vector induced apoptosis *in vitro*. We then determined the effects of intratumoral injection of *bcl-x$_s$* adenovirus on solid MCF-7 tumors in nude mice. Tumors injected four times with the *bcl-x$_s$* adenovirus showed a 50% reduction in size. Using terminal transferase-mediated dUTP-digoxigenin nick end labeling, we observed apoptotic cells at sites of *bcl-x$_s$* adenoviral injection. These experiments demonstrate the feasibility of using *bcl-x$_s$* gene therapy to induce apoptosis in human breast tumors.

Introduction

Overexpression of genes that inhibit programmed cell death (apoptosis) may play a role in the etiology of a variety of cancers (1), including breast cancer. Genes in these pathways, therefore, may represent novel targets for cancer therapy. The proto-oncogene *bcl-2* is overexpressed in up to 70% of breast cancers (2) and may be an important negative regulator of apoptosis in these cancers. The Bcl-2 protein was first described as being overexpressed in follicular lymphomas, in which it participates in tumor formation by blocking cell death (3, 4). The ability of Bcl-2 overexpression to inhibit apoptosis has been verified *in vivo* using transgenic mice (5). A genetic homologue of *bcl-2* named *bcl-x* has been cloned. Due to alternate mRNA splicing, the *bcl-x* gene is transcribed into long (*bcl-x$_L$*) and short (*bcl-x$_s$*) forms (6). The Bcl-x$_L$ protein, like Bcl-2, functions as an inhibitor of apoptosis (6–8). Overexpression of Bcl-x$_L$ has been shown to protect human neuroblastoma cells from apoptosis induced by the chemotherapeutic agents 4-hydroperoxycyclophosphamide and cisplatin (7). In contrast, the Bcl-x$_s$ protein functions as a dominant negative repressor of Bcl-2 and Bcl-x$_L$ (6, 9). We have previously reported that 4-fold overexpression of Bcl-x$_s$ in MCF-7 human breast cancer cells, which express Bcl-2 and overexpress Bcl-x$_L$, sensitized these cells to apoptosis induced by the chemotherapeutic agents etoposide and taxol (10). The inability to produce stably transfected clones that overexpress Bcl-x$_s$ by more than 4-fold suggests that higher levels of Bcl-x$_s$ may be lethal to cells. Therefore, we hypothesized that transient overexpression of large amounts of Bcl-x$_s$ would induce apoptosis in the absence of other insults. To test this, we used adenovirus-mediated transfer of a *bcl-x$_s$* minigene to transiently overexpress high levels of Bcl-x$_s$ in MCF-7 cells. We report that this transduction caused apoptosis *in vitro*. In other systems, such as prostate and head and neck cancer, adenoviral-mediated transfer of the tumor suppressor genes *p53* and *p21* have been shown to inhibit solid tumor growth *in vivo* (11, 12). Based on our *in vitro* data, we have extended our studies to examine the effect of adenovirus-transduced *bcl-x$_s$* on MCF-7 tumor growth *in vivo*. We report that such *bcl-x$_s$* gene therapy retards the growth of solid MCF-7 tumors in nude mice by the induction of apoptosis *in vivo*.

Materials and Methods

Construction of *bcl-x$_s$* and *lacZ* Adenoviruses. The *bcl-x$_s$* minigene was excised from the plasmid pBS*bcl-x$_s$* (provided by G. Nuñez; Ref. 6). The bacterial *lacZ* gene (with nuclear localization sequence) or *bcl-x$_s$* minigene was inserted into the *Bam*HI site of the pAd5RSV plasmid. This plasmid was derived by inserting the RSV[3] promoter into the *Bgl*2 site of the pAdBgl2 vector.[4] The *bcl-x$_s$* and *lacZ* recombinant adenoviruses were isolated by *in vivo* homologous recombination between the pAd5RSV*bcl-x$_s$* or pAd5RSV*lacZ* plasmid and replication-deficient sub360 adenovirus, which has a deletion of the *E1A* and *E1B* genes, as described (9). Large preparations of the adenovirus were made by infecting 293 kidney cells and purifying crude virus by cesium chloride centrifugation. The viral titer was quantified by spectrophotometry.

Growth Studies of MCF-7 Cells. MCF-7 human breast cancer cells were cultured in MEM plus L-glutamine (Celox, Hopkins, MN) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acid solution (Sigma Chemical Co., St. Louis, MO). For growth studies, MCF-7 cells were first plated at 10^4 cells/well for 24 h, then treated with serum-free MEM containing the *bcl-x$_s$* or *lacZ* adenovirus at 2–5 × 10^3 pfu/cell for 3 h. This medium was replaced with MEM plus insulin plus 4% fetal bovine serum overnight, then full culture medium. Cell growth was assessed by cell counts using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and by hemocytometry.

Western Blot Analysis. Protein samples were resolved by denaturing SDS-PAGE and Western blotted by standard methods. Bcl-x$_L$ and Bcl-x$_s$ were detected using a rabbit polyclonal IgG to human Bcl-x (Santa Cruz Biotechnology, Santa Cruz, CA). A goat antirabbit antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham, Arlington Heights, IL) were used to visualize protein bands.

Quantitation of Apoptosis by ELISA. To quantitate the relative number of apoptotic cells *in vitro*, we used an ELISA (Boehringer Mannheim, Indianapolis, IN), which measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, as described previously (10).

Growth of MCF-7 Solid Tumors in Nude Mice. Six-week-old, female, athymic nude mice (CD-1, *nu/nu*; Charles River Breeding Laboratories, Wilmington, MA) were implanted with 0.72 mg 17β-estradiol pellets (6-week time release; Innovative Research of America, Sarasota, FL). Two days later, 6 × 10^6 MCF-7 cells were injected s.c. in 0.1 ml 50% matrigel (Collaborative Biomedical Products, Bedford, MA) plus 50% unsupplemented MEM, as described (13). This method has yielded a 95% success rate for the formation of MCF-7 tumors *in vivo*.

Adenoviral-mediated Bcl-x$_s$ Overexpression *in Vivo*. One of two tumors per mouse was treated by direct intratumoral injection with the *bcl-x$_s$* adenovirus, and the other was injected with the *lacZ* adenovirus, using a 0.1-ml syringe with a 26-gauge, 0.5-inch needle. A single needle puncture was made into the skin covering the tumor; then, the needle tip was moved to five

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[3] The abbreviations used are: RSV, Rous sarcoma virus; pfu, plaque forming unit; gal, galactosidase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling.

[4] B. Davidson, personal communication.

different sites within the tumor, and 10 μl adenovirus preparation were injected at each site. Tumor volume was measured every 3–4 days. Linear calipers were used to measure the longest axis (*a*) and the width perpendicular to this axis (*b*). Tumor volume was calculated as: volume = $a \times b^2 \times 0.4$.

X-gal Staining and Detection of Apoptosis by TUNEL. Mice were sacrificed, and tumors were frozen in OCT embedding medium (Miles, Inc., Elkhart, IN) by standard methods. β-Gal activity was detected by X-gal staining on 20-μm cryosections as described (14). Internucleosomal DNA cleavage characteristic of apoptotic cells was detected *in situ* on 8-μm cryosections by TUNEL (ApopTag kit; Oncor, Gaithersburg, MD) as described (15).

Statistics. For ELISA data and *in vitro* cell counts, statistical significance was calculated by Student's paired *t* test. For *in vivo* tumor growth experiments, overall statistical significance between control and treated groups was calculated by repeated-measures analysis using Statistical Analysis System software (SAS Institute, Inc., Cary, NC). *P* values are given in each figure legend.

Results

Overexpression of Bcl-x_s by an Adenoviral Vector Induces Apoptosis of MCF-7 Human Breast Cancer Cells *in Vitro*. Expression of Bcl-x family members by MCF-7 cells was assayed by Western blotting using a rabbit polyclonal antibody that recognizes both Bcl-x_L (M_r 25,630) and Bcl-x_s (M_r 18,700). As shown in Fig. 1*A*, *Lanes 3* and *4*, control MCF-7 cells exclusively express Bcl-x_L and do not express Bcl-x_s. The effects of transient overexpression of Bcl-x_s on MCF-7 cells *in vitro* were tested by infecting these cells with an adenovirus containing a *bcl-x_s* minigene (pAdRSV-*bcl-x_s*). Another adenovirus carrying the bacterial gene for β-gal (pAdRSV-*lacZ*) was used as a control. As shown in Fig. 1*A*, *Lanes 1* and 2, MCF-7 cells infected 3 days previously with 2×10^3 pfu/cell *bcl-x_s* adenovirus express Bcl-x_s in addition to Bcl-x_L.

We next determined the effect of Bcl-x_s overexpression on cell viability in culture. Beginning 2 days after infection, large numbers of dead, floating cells were observed in MCF-7 cultures infected with the *bcl-x_s* adenovirus. Fig. 1*B* shows that 5 days after infection with 10^4 pfu/cell, the *bcl-x_s* adenovirus caused a 45% greater reduction in the number of viable cells, as determined by trypan blue exclusion, than the *lacZ* adenovirus ($P = 0.010$; $n = 3$). To determine whether these differences in viable cell numbers were caused by apoptosis induced by the *bcl-x_s* adenovirus, we used an ELISA. This assay quantitates cytoplasmic, histone-bound DNA derived from internucleosomal DNA fragmentation, which occurs in the nuclei of apoptotic cells. Table 1 shows that cells infected with 2×10^3 pfu/cell *bcl-x_s* adenovirus had 6-fold greater cytoplasmic DNA-histone than uninfected controls and 4.6-fold greater DNA-histone than *lacZ* controls ($P \leq 0.01$; $n = 3$). These data suggest that the *bcl-x_s* adenovirus, specifically, induces apoptosis in MCF-7 cells, accounting for the observed decrease in viability.

We then tested whether the MCF-7 cells which remained viable by trypan blue exclusion after *bcl-x_s* infection were clonogenic. To accomplish this, we tested the ability of *bcl-x_s*- or *lacZ*-infected MCF-7 cells to grow as solid, s.c. tumors in estrogen-supplemented nude mice. Three $\times 10^7$ pfu *bcl-x_s* or *lacZ* adenovirus were added to 3×10^6 MCF-7 cells immediately prior to s.c. injection into the flank of a mouse. Fig. 1*C* shows that although the *bcl-x_s*-infected cells formed a small nodule, the nodule did not grow ($P = 0.0085$; $n = 2$). The *lacZ*-infected cells formed larger nodules, which, after a growth delay, began to grow at a rate similar to that in uninfected controls. After 25 days, *bcl-x_s*-infected tumors were 91% smaller than *lacZ*-infected controls. These results indicate that the MCF-7 cells which excluded trypan blue following *bcl-x_s* infection were incapable of growing as a tumor *in vivo*, suggesting that these cells were no longer clonogenic. When titers higher than 3×10^7 pfu were tested, the adenovirus vector alone was toxic to the cells, and tumors did not form.

***In Vivo bcl-x_s* Adenoviral Treatment Retards the Growth of MCF-7 Tumors in Nude Mice.** To determine whether the *bcl-x_s* adenoviral vector would be useful for *in vivo* gene therapy, we extended our study to see whether *in vivo bcl-x_s* adenoviral treatment would retard the growth of established tumors. Three-day-old MCF-7 tumors in estrogen-supplemented nude mice were treated by single injections of 7×10^7 pfu *bcl-x_s* or *lacZ* adenovirus. Fig. 2*A* shows that tumors treated with the *bcl-x_s* adenovirus grew at a decreased rate for 8 days after treatment, then began to grow at a rate similar to *lacZ*-infected tumors ($P = 0.031$; $n = 3$). Tumors treated with the *lacZ* adenovirus grew at a rate similar to that of untreated controls. The greatest *bcl-x_s*-specific tumor growth inhibition was observed 8

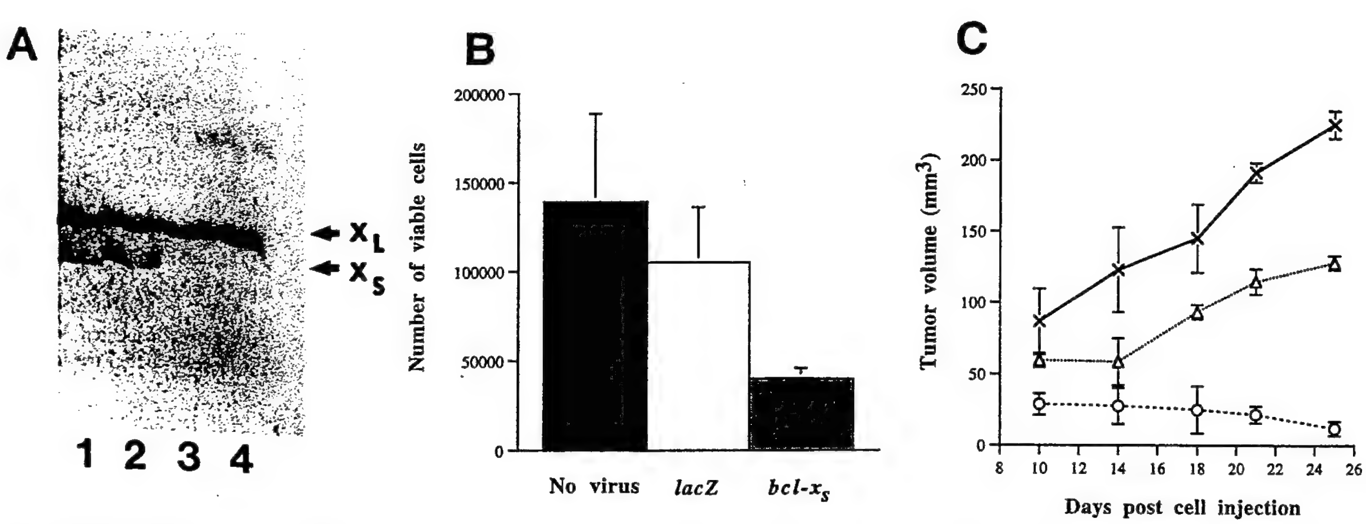

Fig. 1. Effects of Bcl-x_s expression on cell viability. *A*, Western blot of Bcl-x expression. MCF-7 cells were infected as described in "Materials and Methods" with 2×10^3 pfu/cell *bcl-x_s* or *lacZ* adenovirus, and total protein lysates (400 μg/lane) were analyzed by Western blotting using a rabbit polyclonal antibody to Bcl-x. *Lanes 1* and 2, *bcl-x_s*-infected cells; *lanes 3* and *4*, *lacZ*-infected cells. *Arrows:* x_L, Bcl-x_L (M_r 25,630); x_s, Bcl-x_s (M_r 18,700). *B*, effect of *bcl-x_s* adenoviral infection on MCF-7 viability. MCF-7 cells were infected *in vitro* with 10^4 pfu/cell *bcl-x_s* or *lacZ* adenovirus. The number of live cells, as determined by trypan blue exclusion, was counted 5 days later ($P = 0.010$; *bars*, SE; $n = 3$). *C*, effect of *bcl-x_s* adenoviral infection on clonogenicity of MCF-7 cells. MCF-7 cells (3×10^6) were treated with 10 pfu/cell *bcl-x_s* or *lacZ* adenovirus *in vitro* and injected s.c. into opposite flanks of an estrogen-supplemented nude mouse. Tumor volume was calculated as described in "Materials and Methods" ($P = 0.0085$; *bars*, SD; $n = 2$). ×, no virus; △, *lacZ*; ○, *bcl-x_s*.

Table 1 *Quantitation of apoptosis by ELISA*

MCF-7 cells were infected with the *bcl-x_s* or *lacZ* adenovirus (2×10^3 pfu/cell), and after 6 days, cytoplasmic extracts from equal total numbers of cells (live and dead) were assayed for DNA-histone as described (10). Values are normalized to 1.0 for uninfected MCF-7 cells ($P \leq 0.01$; $n = 3$).

Sample	Mean ± SE units of cytoplasmic DNA-histone
Uninfected MCF-7	1.00
bcl-x_s-infected MCF-7	6.07 ± 1.94
lacZ-infected MCF-7	1.32 ± 0.25

days after treatment, when *bcl-x_s*-infected tumors were 38% smaller than *lacZ*-infected tumors. This experiment showed that *in vivo bcl-x_s* adenoviral infection retarded tumor growth, but that single treatment was not sufficient for long-term growth inhibition.

We hypothesized that, relative to *in vitro* infection, *in vivo* infection had less effect on tumor growth because only a fraction of the cells was infected; this hypothesis was subsequently confirmed (see Fig. 3*A*). Therefore, we tested whether multiple *in vivo* infections with the *bcl-x_s* adenovirus would cause greater or sustained retardation of tumor growth. Three-day-old MCF-7 tumors were treated with 7×10^7 pfu *bcl-x_s* or *lacZ* adenovirus every 4 days for five treatments. Fig. 2*B* shows that throughout the duration of treatment, the *bcl-x_s*-infected tumors grew at a slower rate than the *lacZ*-infected tumors ($P = 0.0032$; $n = 4$). The greatest *bcl-x_s*-specific retardation of growth was observed following four treatments (13 days after the first treatment), when *bcl-x_s*-infected tumors were 52% smaller than *lacZ*-infected tumors. Thus, multiple treatments with the *bcl-x_s* adenovirus caused greater and sustained tumor growth inhibition relative to single treatment.

Inhibition of MCF-7 Tumor Growth *in Vivo* by *bcl-x_s* Adenoviral Infection Is Due to Induction of Apoptosis. To determine the efficacy of our direct intratumoral approach to adenoviral-mediated gene therapy, we estimated the number of cells in an infected tumor that expressed the transduced gene, using *lacZ* as a marker. Three-day-old MCF-7 tumors were treated *in vivo* with 7×10^7 pfu *lacZ* adenovirus, and expression of the viral-encoded gene was detected 4 days later by X-gal staining. Overall, about 15% of the tumor cells expressed the transduced *lacZ* gene ($n = 2$). As seen in Fig. 3*A*, we found a higher concentration of transduced cells along linear tracks, presumably at sites of needle injection. Thus, gene transfer was successful to a portion of the tumor cells.

To determine whether cells infected *in vivo* with the *bcl-x_s* or *lacZ* adenovirus were apoptotic, X-gal staining was used to locate infected cells; then, apoptotic cells were detected on serial sections by TUNEL. Fig. 3*B* shows a TUNEL assay of a serial section of the *lacZ*-infected tumor seen in Fig. 3*A*. The absence of apoptotic cells in the infected region of the tumor shows that the *lacZ* adenovirus did not induce apoptosis ($n = 2$). To determine whether the *bcl-x_s* adenovirus induced apoptosis in transduced cells, we used a combined approach, in which 90% *bcl-x_s* adenovirus was mixed with 10% *lacZ* adenovirus as a tracer. This approach allowed us to determine areas of successful gene transfer using *lacZ* as a marker while analyzing the effects of *bcl-x_s* on apoptosis. Fig. 3, *C* and *D*, shows X-gal staining and TUNEL of a tumor treated *in vivo* with 90% *bcl-x_s* plus 10% *lacZ* adenovirus. In contrast to treatment with *lacZ* alone, the addition of the *bcl-x_s* adenovirus caused apoptosis in the infected area of the tumor ($n = 2$). This is seen more clearly in Fig. 3*E*, which shows apoptotic nuclei visible at a higher magnification. These experiments demonstrate that *in vivo bcl-x_s* adenoviral treatment induced cell death by apoptosis, consistent with the inhibition of the overall tumor growth rate caused by this treatment.

Discussion

Normal mammary epithelial cells are capable of undergoing apoptosis during involution following lactation (16). Inhibitors of this ability to undergo apoptosis, such as Bcl-2 and Bcl-x_L, may play an important role in the development of breast cancer. In this communication, we report that MCF-7 human breast cancer cells overexpress Bcl-x_L but do not express Bcl-x_s. Bcl-x_s is a dominant negative repressor of Bcl-2 and Bcl-x_L and is, therefore, a putative inducer of apoptosis in cancer cells which are resistant to apoptosis due to overexpression of Bcl-2 and/or Bcl-x_L (6, 9). We report that adenovirus-mediated overexpression of Bcl-x_s induces apoptosis of MCF-7 human breast cancer cells *in vitro*. In addition, we report that adenovirus-mediated *bcl-x_s* gene therapy can retard MCF-7 tumor growth in a nude mouse model and present evidence that this growth inhibition is due to induction of apoptosis. The *bcl-x_s* adenovirus, which is replication deficient, caused no obvious systemic or local toxicity, such as skin necrosis, when delivered locally.

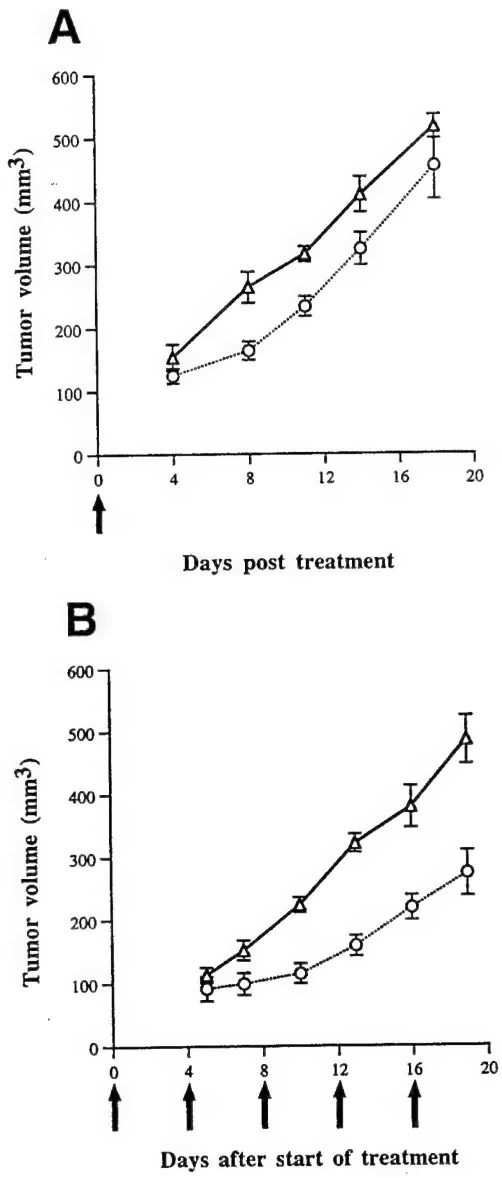

Fig. 2. *bcl-x_s* adenoviral effects on tumor growth *in vivo*. *A*, tumor growth following single injection. Three days after s.c. injection of 6×10^6 MCF-7 cells, tumors were injected with 7×10^7 pfu *bcl-x_s* or *lacZ* adenovirus ($P = 0.031$; *bars*, SE; $n = 3$). *B*, tumor growth following multiple injections. Tumors were treated five times, once every 4 days ($P = 0.0032$; *bars*, SE; $n = 4$). *Arrows*, treatment. △, *LacZ*; ○, *bcl-x_s*.

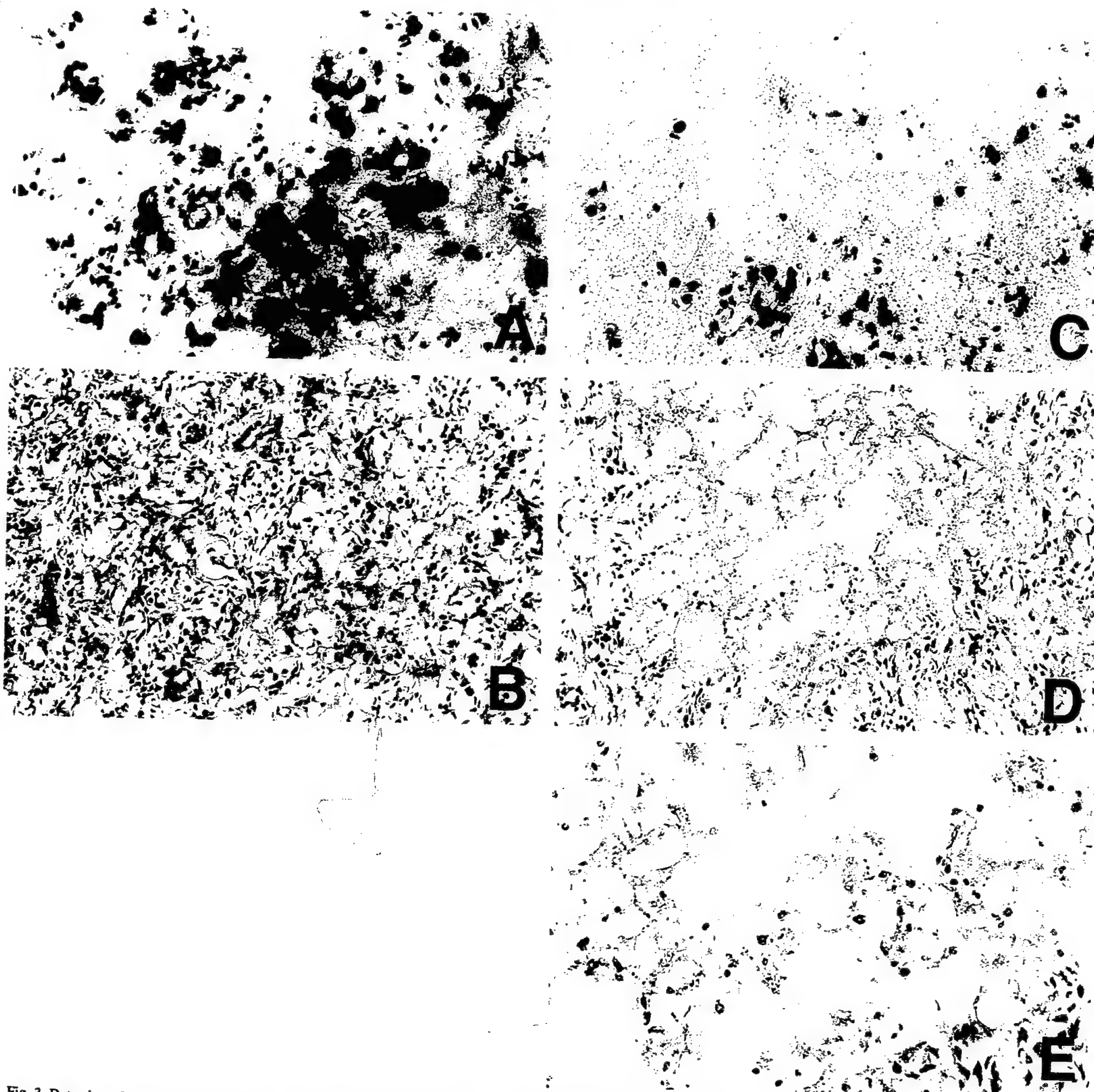

Fig. 3. Detection of gene expression and apoptosis *in situ*. Three-day-old MCF-7 tumors were treated with *lacZ* with or without $bcl\text{-}x_s$ adenovirus (7×10^7 pfu total) as indicated ($n = 2$). Four days later, mice were sacrificed, and tumors were assayed for *lacZ* gene transfer by X-gal staining (blue) and apoptosis by TUNEL (apoptotic cells appear brown). *A*, 100% *lacZ*, X-gal staining. *B*, 100% *lacZ*, TUNEL. *C*, 90% $bcl\text{-}x_s$ plus 10% *lacZ*, X-gal staining. *D*, 90% $bcl\text{-}x_s$ plus 10% *lacZ*, TUNEL. *E*, higher magnification of *D*.

In vivo $bcl\text{-}x_s$ adenoviral treatment reduced the size of the solid MCF-7 tumors by a greater amount than would be predicted from the percentage of cells that were infected as determined by *lacZ* expression. Single *in vivo* adenoviral treatment resulted in $bcl\text{-}x_s$-infected tumors that were 38% smaller than *lacZ*-infected controls. However, X-gal staining showed that a single adenoviral treatment infected only about 15% of the cells in each tumor. This raises the possibility that $bcl\text{-}x_s$ adenoviral infection kills additional cells via a bystander effect, in which uninfected cells surrounding an infected cell also are killed. The bystander effect has been shown to induce apoptosis of unmodified tumor cells adjacent to cells transduced with the herpes simplex virus thymidine kinase gene and treated with ganciclovir (17). Such bystander killing was associated with the transfer of apoptotic vesicles from transduced, dying cells to adjacent untransduced cells (17). We are currently investigating whether $Bcl\text{-}x_s$-induced apoptosis may cause a similar bystander effect.

The *in vitro* ELISA data and *in situ* TUNEL assays presented here suggest that a significant fraction of the cell killing caused by the $bcl\text{-}x_s$ adenovirus is due to apoptosis. It is not yet clear whether this apoptosis is solely the result of $Bcl\text{-}x_s$ overexpression, or whether the adenoviral vector presents an insult to the cell which contributes to the induction of apoptosis. However, the $bcl\text{-}x_s$ adenovirus caused significantly greater apoptosis than the same titer of the *lacZ* adenovirus, providing evidence that $Bcl\text{-}x_s$ overexpression itself contributes to apoptosis and showing the importance of blocking inhibitors of cell death, such as Bcl-2 and $Bcl\text{-}x_L$. Furthermore, this shows that adeno-

viral-mediated gene transfer of *bcl-x_s*, which causes transient overexpression, is sufficient to induce apoptosis.

A possibility for improving the efficacy of *bcl-x_s* gene therapy may come from a combination of this approach with chemotherapy. We have previously reported that stable transfection of *bcl-x_s* sensitized MCF-7 cells to apoptosis induced by the chemotherapeutic agents etoposide and taxol *in vitro* (10). By blocking inhibitors of apoptosis, *bcl-x_s* gene therapy may lower the apoptotic threshold of cancer cells to other agents that cause cell death.

Bcl-2 and Bcl-x_L, the functional targets of Bcl-x_s, seem to inhibit apoptosis through a common pathway (18). Bcl-2 has been shown to be overexpressed in up to 70% of breast cancers (2) and, therefore, may play an important role in the development of these cancers. Although such data have not yet been reported for Bcl-x_L, we have found Bcl-x_L to be overexpressed in a significant percentage of primary cell lines and tissues derived from breast cancers (data not shown). These data suggest that Bcl-2 and Bcl-x_L may be important targets for novel breast cancer treatments, such as *bcl-x_s* adenoviral gene therapy.

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MANUSCRIPT #4
From manuscript and abstract list in references

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Bcl-x_S Enhances Adenoviral Vector-induced Apoptosis in Neuroblastoma Cells[1]

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ABSTRACT

***bcl-x* is a member of the *bcl-2* family of genes and by alternative splicing gives rise to two distinct mRNAs: *bcl-x_L* and *bcl-x_S*. We have previously investigated the expression of Bcl-x in neuroblastoma (NB) cell lines and have shown that Bcl-x_L is expressed and functions to inhibit chemotherapy-induced apoptosis. However, none of the NB cell lines expressed Bcl-x_S. The aim of the present study was to determine the effects of Bcl-x_S expression on the viability of NB cells. A panel of NB cell lines (CHP-382, GOTO, SHEP-1, SHSY-5Y, and GI-CA-N) were infected with either a *bcl-x_S* adenovirus (pAdRSV-*bcl-x_S*) or a control virus (pAdRSV-*lac-z*). NB cells showed loss of viability with both viruses, although the *bcl-x_S* virus was most toxic. Importantly, infection with the *bcl-x_S* adenovirus resulted in rapid loss of cell viability, DNA fragmentation, and morphological features of apoptosis even in NB cells transfected to overexpress Bcl-2 and Bcl-x_L. These findings suggest that deregulated expression of Bcl-x_S using an adenovirus may provide a novel mechanism for initiating cell death in tumors that express Bcl-2 or Bcl-x_L.**

INTRODUCTION

PCD[3] or apoptosis is an essential feature of the regulation of eukaryotic cell number. It is characterized by the activation of an intrinsic genetic program leading to typical morphological changes, such as cytoplasmic contraction, plasma membrane blebbing, nuclear condensation, and DNA degradation, culminating in cell death (1). The biochemical hallmark of this process in epithelial cells is high molecular weight DNA fragmentation best demonstrated by PFGE (2, 3). *bcl-2* was the first member of a family of genes, the products of which function to modulate PCD pathways. Initially identified at the t(14;18) translocation in follicular B-cell lymphomas (4), subsequent work showed that overexpression could promote cell survival (5) by inhibiting PCD induced by growth factor withdrawal (6). A number of studies by numerous investigators have now determined that Bcl-2 delays or prevents PCD induced by a variety of stimuli, including growth factor deprivation, γ-radiation, glucocorticoids, heat shock, and multiple chemotherapeutic agents (5, 7–9).

bcl-x is a new member of the *bcl-2* family of genes and was isolated by cross hybridization with a *bcl-2* probe (10; reviewed in Ref. 11). As a result of alternative splicing, this gene gives rise to two distinct mRNAs designated as *bcl-x_S* and *bcl-x_L*. The larger transcript encodes a 241-amino acid protein (Bcl-x_L), which displays a high level of amino acid and structural homology to Bcl-2. Bcl-x_L, like Bcl-2, functions to inhibit PCD induced by growth factor withdrawal in hematopoietic cells (10). The smaller transcript, *bcl-x_S*, encodes a 178-amino acid protein, which lacks an internal 63-amino acid domain in the region of highest homology to Bcl-2. In single-gene transfection experiments in hematopoietic cells, Bcl-x_S had no effect on cell growth in the presence of growth factor but facilitated PCD by inhibiting the death-suppressing activity of Bcl-2 (10). Boise *et al.* (10) have proposed that Bcl-x_S acts in a dominant fashion by forming inactive heteromeric complexes with Bcl-2 or Bcl-x_L. In support of this hypothesis are studies by Sato *et al.* (12) showing that Bcl-x_L and Bcl-x_S can combine to form heterodimers or heteromultimers with Bcl-2 in a yeast two-hybrid system. Recent studies have indicated that Bcl-x_L is widely expressed (13, 14), and the pattern of expression varies from that of Bcl-2, and other *bcl-2* gene family members (14–16). The Bcl-x_S protein was detected in the thymus, lymph nodes, tonsils, and reproductive tissues such as testes, prostate, and mammary epithelia. Bcl-x_S levels were variable, and in some tissues, both Bcl-x_L and Bcl-x_S were detected, suggesting a hypothesis that the ratio of Bcl-x_L:Bcl-x_S may modulate PCD induction *in vivo* (13).

Our laboratory has been investigating whether a failure of PCD contributes to the genesis or progression of the pediatric malignancy NB. In previous studies, we have determined that the majority of NB cell lines express Bcl-2 and Bcl-x_L and that deregulated expression of these gene products confers resistance to a variety of chemotherapeutic agents (17, 18). Most importantly, none of the NB cell lines expressed Bcl-x_S. The aim of the present study was to determine whether expression of Bcl-x_S could induce apoptosis in NB cells. Using an adenoviral *bcl-x_S* expression vector, we were able to induce apoptosis in NB cells even in the context of high levels of Bcl-2 and Bcl-x_L expression. Analysis of cells expressing high levels of Bcl-x_S revealed high molecular weight DNA degradation and morphological features of apoptosis. These results suggest that deregulated Bcl-x_S expression using an adenoviral expression system can abrogate the protective effects of Bcl-2 and Bcl-x_L and may provide a novel mechanism for increasing the sensitivity of NB cells to PCD.

MATERIALS AND METHODS

Cell Lines. NB cell lines used in the following study included CHP-382, GI-CA-N, GOTO, SH-SY-5Y, and SHEP-1. Infection studies were also performed in SHEP-1 cells transfected to express high levels of Bcl-2 or Bcl-x_L. These lines included vector control (pooled SHEP-1 cells transfected with the pSFFVneo expression vector alone), SHEP-1 Bcl-2^+ (a cloned cell line transfected with the expression vector pSFFV-*bcl-2*), and SHEP Bcl-x_L^+ (a cloned cell line transfected with the expression vector pSFFV-*bcl-x_L*). The method of transfection and the characterization of the *in vitro* growth of the transfected cell lines has been previously reported (17, 18). Cells were maintained in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Transfected cells were maintained in the same media supplemented with G418 (Geneticin; Life Technologies, Inc., Gaithersburg, MD) at 500 μg/ml.

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[3] The abbreviations used are: PCD, programmed cell death; NB, neuroblastoma; PFGE, pulsed field gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VCI, viable cell index; FACS, fluorescence-activated cell sorting; RSV, Rous sarcoma virus; IL-3, interleukin 3; Topo II, topoisomerase II; SFFV, spleen focus forming virus.

Preparation of Adenovirus and NB Cell Infection. The *bcl-x_S* adenoviral vector (pAdRSV-*bcl-x_S*; Ref. 19) was constructed by cloning a full-length *bcl-x_S* cDNA into the pAdRSV expression vector (20). This vector uses an RSV promoter and SV40 polyadenylation signal, allowing high-level expression of inserted sequences. Recombinant adenovirus was produced by cotransfecting pAdRSV-*bcl-x_S* with the replication-deficient sub360 adenovirus into 293 human kidney cells (20). Viral titers were determined by limiting dilution and plaque formation of 293 cells. Each infection was performed in duplicate with at least three viral stock preparations. Controls for these experiments included a mock infection (no virus) and infection with the same adenovirus construct containing *lac-z* (pAdRSV-*lac-z*; Ref. 19). For adenoviral infection, the cell lines were rinsed with serum-free DMEM before adding virus (pAdRSV-*bcl-x_S* or control virus pAdRSV-*lac-z*). Following a 3-h incubation, the virus-containing medium was replaced with DMEM containing 2% fetal bovine serum and incubated overnight. The next day, the medium was replaced with DMEM containing 10% serum. The virally transduced cells were grown for an additional 24 h prior to subsequent experimental manipulation. Mock-infected cells were subjected to the same culturing manipulations without the addition of virus-containing medium.

Detection of Bcl-x_S Expression by Western Analysis. Bcl-x_S was identified by Western analysis using the enhanced chemiluminescence technique (Amersham International, Buckinghamshire, United Kingdom). Twenty-four h following infection, cells were harvested into lysis buffer containing 50 mM Tris, 2% SDS, and 10% glycerol, boiled for 10 min, and centrifuged at $10,000 \times g$. After determining protein concentration, 20 μg of protein were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose by electroblotting. Membranes were blocked and then incubated with an anti-Bcl-x polyclonal antibody that is known to recognize both Bcl-x_L and Bcl-x_S (21). Following incubation with a rabbit secondary antibody, antigen-antibody complexes were detected by exposing the membrane to equal volumes of reagents 1 and 2 from the enhanced chemiluminescence kit. Blots were developed by exposing the membrane to Kodak XAR film. Controls for Western analysis included a NB cell line transfected to express Bcl-x_L (17) and a mouse cell line transfected to express Bcl-x_S (FL5.12 *bcl-x_S*-Ha tag; Ref. 16).

Cell Viability. *In vitro* cell viability was determined by the MTT dye reduction assay (22). Following infection, 10^4 cells were plated in triplicate in 96-well microtiter plates overnight. Plates were harvested daily for 7 days. Media were removed and replaced with 125 μl of complete media containing 1 mg/ml MTT. Plates were incubated at 37°C for 4 h. Following incubation, cells were lysed in a buffer containing 20% (w/v) SDS and 50% (v/v) *N,N*-dimethylformamide (pH 4.5). Absorbance at 570 nM was determined for each well using an MR 650 96-well multiscanner (Dynatech Laboratories, Inc.). After subtracting the background absorbance, the relative number of viable cells compared with the start of treatment was expressed as the VCI [VCI = (A_{570} virus or mock)/(A_{570} day 0) $\times$ 100]. The day-0 reading was obtained 6 h after cells were counted and plated onto the 96-well dishes. Student's paired *t* test was used to test the significance of the difference in cell viability following infection with control virus (pAdRSV*lac-z*) or *bcl-x_S* virus (pAdRSV-*bcl-x_S*).

FACS Analysis for DNA Damage. FACS analysis of propidium iodide-stained nuclei was performed as described previously (23). This assay is rapid and provides an excellent method for assessing DNA damage in apoptotic cells (24). Cells were plated at a density of 1×10^6 cells/well in six-well dishes overnight. At indicated time points, cells were harvested by trypsinization, suspended in hypotonic lysing buffer (0.1% sodium citrate, 0.01% Triton X-100, and 0.1 mg/ml propidium iodide) and incubated at 4°C for 4 h. Cells were analyzed for DNA content on FACS (Becton Dickinson, Mountain View, CA) using Lysis-II software (Becton Dickinson). Differences in percentage of DNA fragmentation between *lac-z*- and *bcl-x_S*-infected cells was determined using Student's paired *t* test.

PFGE Analysis for DNA Fragmentation. In addition to FACS analysis, DNA integrity was also assessed by PFGE. This technique has been shown to correlate with morphological changes (3) and is capable of detecting the early phases of DNA degradation in apoptotic cells (25). Following infection, cells were harvested by trypsinization, and pellets were prepared in low-melting point agarose as described previously (17). Cell pellets were loaded into 1.2% agarose gels (Fastlane; FMC Bioproducts, Rockland, ME) and run at 6 V/cm with ramped switch times from 26 to 30 s over 20 h using the Chef DR-III system apparatus (Bio-Rad Laboratories, Hercules, CA). Following electrophoresis, gels were stained in ethidium bromide and destained in distilled water. λ DNA digested with *Hin*dIII (Life Technologies) and *Coliphage* λ DNA concatamers (Megabase II; Life Technologies) were used as size standards.

Preparation of Monolayer Cells for Electron Microscopy. Following infection, cells were harvested by trypsinization and washed in serum-free media. Cells were pelleted by centrifugation and in an equal volume suspended in 25% BSA and 6% glutaraldehyde. Pellets were isolated, cut into cubes, and fixed in 0.1 M sodium cacodylate buffer (pH 7.4). Fixed cubes were dehydrated in increasing concentrations of ethanol. Cubes were polymerized in Epon resin (44.5% poly/bed 812 resin, 24.1% dodecenylsuccinic anhydride, 29.8% nadic methyl anhydride, and 1.6% 2, 4, 6-Tri (dimethylaminomethyl)phenol; Polysciences, Inc.) at 60°C overnight. Seventy- to 90-nm ultrathin sections were cut on a Reichert-Jung Ultracut E-43 microtome, placed on copper grids, and stained with 4% uranyl acetate in 50% methanol and Reynolds lead citrate. Sample sections were examined in a Phillips Electronics CM-100 transmission electron microscope. Images were digitally recorded using a Kodak Megaplus 1.6 camera with Advanced Microscopy Techniques Digital Montaging System software. Representative grids were scored, and the percentage of apoptotic cells displaying cytoplasmic (retraction, condensation, and organelle segregation) and nuclear changes (folding, segmentation, chromatin condensation, margination, and nuclear membrane degeneration) was determined at 24 and 72 h.

RESULTS

Bcl-x_S Expression in NB Cells. Previous studies have indicated that NB cell lines including SHEP-1 do not express endogenous Bcl-x_S (17). To determine the effects of Bcl-x_S expression in NB cells, we used an adenoviral expression vector to overexpress Bcl-x_S in the NB cell lines CHP-382, GI-CA-N, GOTO, SH-SY-5Y, and SHEP-1. In a separate set of experiments, SHEP-1 cells transfected to express high levels of Bcl-2 or Bcl-x_L were also infected with the Bcl-x_S adenovirus. These lines were chosen because previous studies had determined that deregulated expression of Bcl-2 and Bcl-x_L in SHEP-1 cells could inhibit apoptosis induced by a variety of chemotherapeutic agents, and also that these cells were relatively resistant to the toxic effects of the virus alone. The pAdRSV-*bcl-x_S* expression vector is a replication-incompetent virus that has an infection efficiency of greater than 99%, as determined by staining for β-galactosidase (19). The relative efficiency of adenoviral infection was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining after inoculation of NB target cells with a *lac-z* virus. For each line, viral titers that would result in >99% infection were used. NB cells were infected either with pAdRSV-*bcl-x_S* or pAdRSV-*lac-z* (adenoviral control) or underwent a mock infection (no virus). A high level of Bcl-x_S protein was detected in pAdRSV-*bcl-x_S*-infected cells 24 h after infection by Western blot analysis (Fig. 1). Mock-infected and *lac-z*-infected cells were negative for Bcl-x_S. Expression of β-galactosidase in control infected cells was confirmed by immunohistochemical analysis (data not shown).

The Adenoviral Vector Promotes NB Cell Death, Which Is Enhanced by Bcl-x_S Expression. Cell viability of virally infected NB cells was determined by the MTT viability assay. This assay relies on the ability of intact mitochondria to reduce the yellow MTT compound to a blue formazan derivative. NB cells infected with the adenoviral vectors exhibited one of three patterns of loss of cell viability. In some cells (CHP-382 and GOTO) the *lac-z* and *bcl-x_S* viruses were equally toxic and resulted in rapid and complete loss of cell viability within 72 h of infection (Fig. 1*A*). A second pattern of cell death was seen in SH-SY-5Y cells. Here, both viruses induced equivalent loss of viability over the duration of the experiment, with the *bcl-x_S* virus inducing the most rapid onset of cell death (Fig. 1*B*). A third pattern was seen in GI-CA-N and SHEP-1 cells. In these cells, the *lac-z* virus caused minimal loss of viability, whereas the *bcl-x_S*

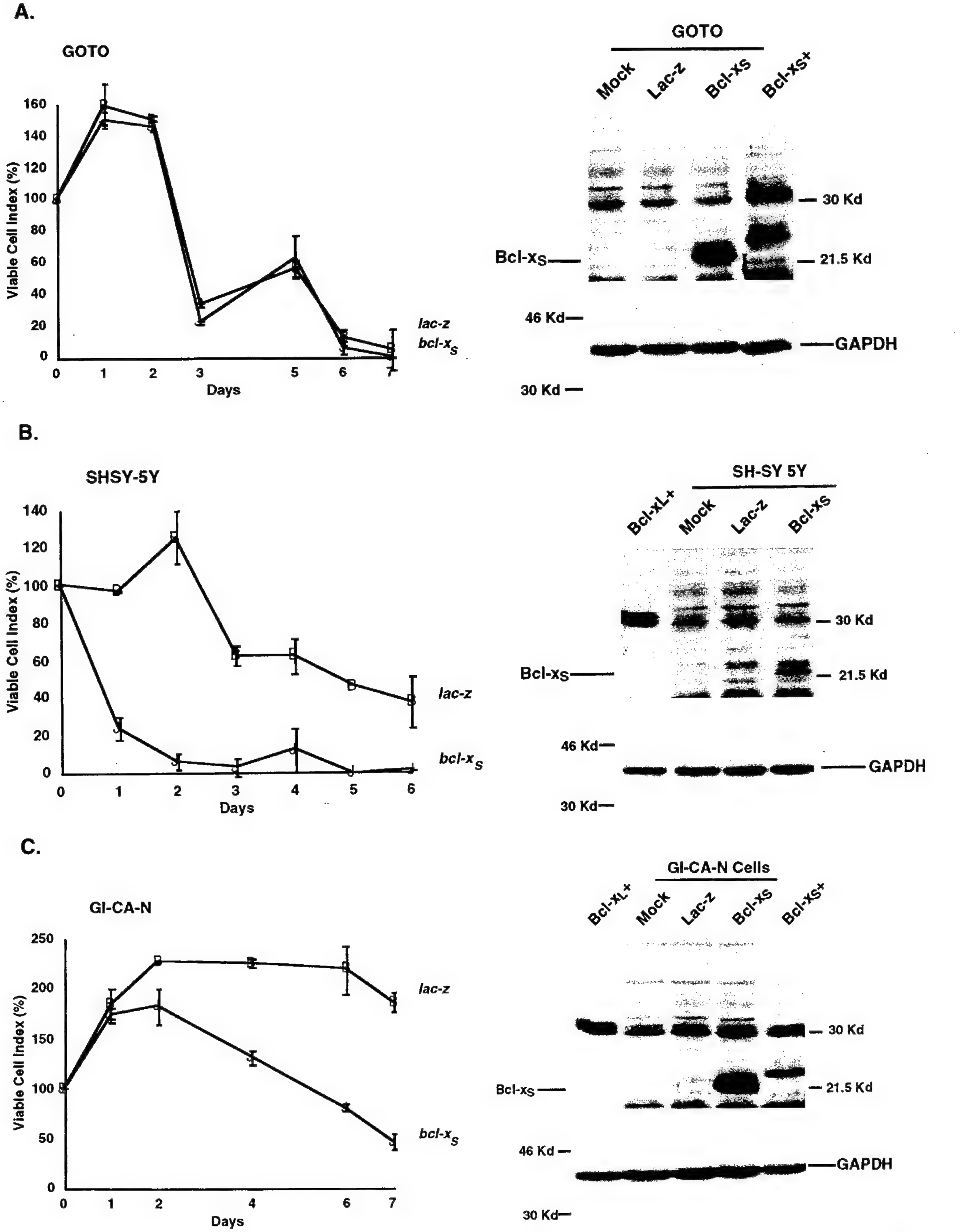

Fig. 1. Cell viabilities of NB cell lines GOTO (*A*), SHSY-5Y (*B*), and GI-CA-N (*C*) infected with the pAdRSV-*lac-z* or pAdRS-*bcl-x_S* virus were determined by MTT conversion. After subtracting background absorbance, the viability of each cell was calculated as VCI = (A_{570} virus or mock)/(A_{570} day 0) $\times$ 100. Data represent the means of triplicate experiments. *Bars,* SD. The VCI for mock-infected cells ranged from 250 to 800% on day 3 (data not shown). Bcl-x_S protein expression following viral infection was confirmed by Western blot. As shown, Bcl-x_S protein was detected in *bcl-x_S* virus-infected cells but not following mock or *lac-z* infection. Positive controls included a cell line transfected to express Bcl-x_L (Bcl-x_L^+) and a cell line transfected to express Bcl-x_S (Bcl-x_S^+-FL5.12 *bcl-x_S*-HA Tag; Ref. 16). The protein product of the Bcl-x_S^+ control migrates higher than the viral induced protein as it expresses a flag tag. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control for protein loading.

virus caused rapid and complete loss of viability within 72 h (Fig. 1*C*). High-level Bcl-x_S protein was detected in all cells 24 h following infection but was absent in *lac-z*-infected or mock-infected cells (Fig. 1).

To determine whether expression of Bcl-2 or Bcl-x_L could overcome the effects of Bcl-x_S expression, SHEP-1 cells transfected to express high levels of Bcl-2 or Bcl-x_L were infected in a similar manner. Similar to control transfected SHEP-1 cells, both the *bcl-*x_S and *lac-z* viruses were inherently toxic to the transfected cell lines regardless of the level of Bcl-2 or Bcl-x_L expression (Fig. 2). The VCI on day 3 following *lac-z* infection was 31% in vector-transfected controls, 81% in Bcl-2-expressing cells, and 146% in Bcl-x_L-expressing cells. On day 5, the VCI in *lac-z*-infected cells was 3% for vector controls, 13% for Bcl-2-expressing cells, and 6% for Bcl-x_L-expressing cells. Importantly, however, cells infected with the *bcl-*x_S adenovirus showed earlier and enhanced loss of cell viability when compared with *lac-z*-infected cells. The VCI on day 3 following *bcl-*x_S infection was 3% ($P < 0.001$) for vector controls, 13% ($P < 0.001$) for Bcl-2-expressing cells, and 30% ($P < 0.002$) for Bcl-x_L-expressing cells. By day 5, all of the *bcl-*x_S-infected cells displayed a VCI of <6%. These results indicate the pAdRSV-*lac-z* virus is toxic to NB cells and that the degree of toxicity is enhanced by expression of Bcl-x_S. Additionally, the *bcl-*x_S adenovirus induces cell death even in the context of high Bcl-2 and Bcl-x_L expression.

Adenoviral *bcl-*x_S Expression Induces Apoptosis with Loss of DNA Integrity and Morphological Evidence of Apoptosis. The biochemical hallmark of apoptosis in SHEP-1 NB cells and other epitheloid cell types is high molecular weight DNA degradation (2, 3, 17, 18). FACS and PFGE analysis were used to assess the integrity of DNA following *bcl-*x_S infection. FACS analysis 24 h following infection revealed that 9–18% of the nuclei contained degraded DNA in *lac-z*-infected cells (Table 1). Once again, the degree of cell toxicity was enhanced following infection with the *bcl-*x_S adenovirus. Vector-transfected controls and the Bcl-2-expressing cells showed a marked loss of DNA integrity, with 49–65% of the nuclei containing degraded DNA ($P < 0.009$).

Analysis of high molecular weight DNA degradation is best demonstrated by PFGE. Twenty-four h following mock and *lac-z* infection, DNA in vector-transfected and Bcl-2- and Bcl-x_L-expressing cells remained intact, with most of the DNA being trapped in the well or in the compression zone immediately below it. In contrast, cells expressing Bcl-x_S showed early loss of DNA integrity with the appearance of high molecular weight DNA (Fig. 3, *A* and *B*). Analysis of DNA integrity 72 h following infection with the *lac-z* virus indicated that the control virus could also induce this pattern of high molecular weight DNA degradation (Fig. 3*C*). These results support the viability assays and suggest that both viruses are toxic to NB cells, but the toxicity is enhanced by Bcl-x_S expression.

The loss in DNA integrity following *lac-z* or *bcl-*x_S infection corresponded to morphological changes consistent with apoptotic cellular demise. As shown in Fig. 4, infection with the *lac-z*-containing virus resulted in no morphological evidence of apoptosis at 24 h (Fig. 4*A*), but by 72 h, >85% of the cells were apoptotic with evidence of chromatin clumping, cytoplasmic retraction, and nuclear membrane degeneration (Fig. 4*B*). In comparison, the *bcl-*x_S virus induced more rapid onset of apoptosis, with 20% of the cells displaying apoptotic features as early as 24 h (Fig. 4*C*). By 72 h, >95% of the cells were apoptotic (Fig. 4*D*). These findings were absent in mock-infected cells (Fig. 4*E*). These results indicate that Bcl-x_S expression enhances the inherent toxicity of the adenoviral vector, resulting in rapid onset of DNA degradation and morphological features of apoptosis.

DISCUSSION

PCD or apoptosis in eukaryotes is a genetically regulated process that can be induced by a variety of stimuli (26). Although a number of different genes have been identified that promote cell death in the nematode *Caenorhabditis elegans* and in humans, the precise biochemical events and control of this process has yet to be elucidated. In previous studies we have determined that a majority of NB cell lines express Bcl-x_L and Bcl-2 and that deregulated Bcl-2 and Bcl-x_L expression can inhibit apoptosis induced by a number of chemotherapeutic agents in these cells (17, 18). Interestingly, none of the NB cell lines expressed Bcl-x_S (18), a Bcl-2 family member that functions

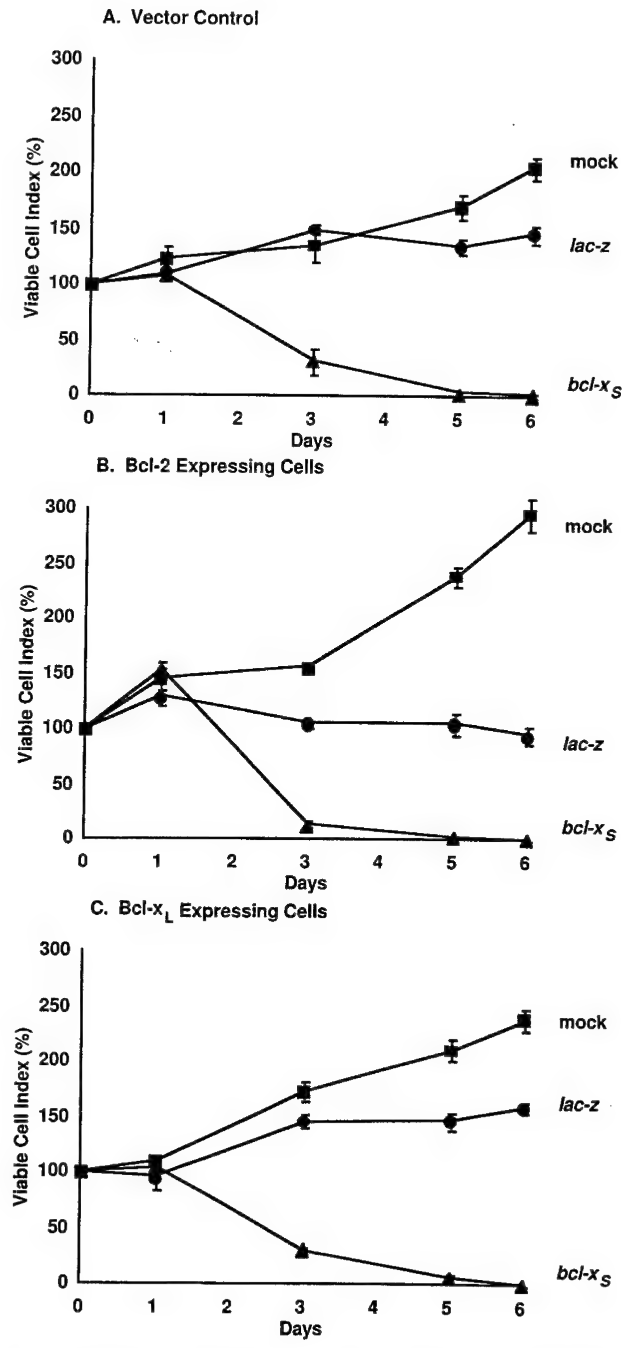

Fig. 2. Cell viabilities were determined by MTT conversion for vector control (*A*), Bcl-2-expressing (*B*), and Bcl-x_L-expressing (*C*) cells following mock, *lac-z*, or *bcl-*x_S infection. Data presented are the mean values of triplicate experiments. *Bars*, SD.

Table 1 *Flow cytometric analysis of DNA from nuclei following mock, pAdRSV-lac-z, or pAdRSV-bcl-x_S infection*

Cell line	Apoptotic cell no. (%)[a]		
	Mock-infected	*lac-z*-infected	*bcl-x_S*-infected
Vector control	9 ± 1	18 ± 2	49 ± 3[b]
Bcl-2-expressing		8.9 ± 2	65 ± 1[c]

[a] Values are mean ± SD.
[b] $P < 0.02$.
[c] $P < 0.009$.

to facilitate apoptosis (10). In this report, we have investigated the effects of adenoviral Bcl-x_S expression on the *in vitro* cell viability and DNA integrity of NB cells, including cell lines that have been transfected to express the apoptosis-suppressing proteins Bcl-x_L or Bcl-2. The results of these studies indicate that the adenoviral vector is inherently toxic to certain NB cells and that expression of Bcl-x_S enhances this toxicity. Importantly, the *bcl-x_S* adenovirus was able to induce rapid onset of apoptosis even in the context of high Bcl-2 or Bcl-x_L expression. Boise *et al.* (10) have shown that expression of Bcl-x_S in IL-3-dependent hematopoietic cells did not affect cell growth in the presence of IL-3 but did abrogate the protective effects of Bcl-2 following growth factor withdrawal in IL-3-dependent hematopoietic cells. In our studies, all cells infected with the *bcl-x_S* adenovirus underwent apoptosis, a result that may be related to the high level of Bcl-x_S expression, which is achieved using an adenoviral expression vector. In support of this interpretation are studies that indicate that MCF-7 cells transfected to express a low level of Bcl-x_S maintain their viability in culture yet show enhanced chemotherapy-induced apoptosis (27). In contrast, the same cells show marked apoptotic death when high-level Bcl-x_S expression is achieved using an adenoviral expression system (19). The mechanism by which the *bcl-x_S* adenovirus induced cell death is unknown. It has been shown that Bcl-x_S fails to interact with Bcl-x_L and Bcl-2 *in vivo*, suggesting that Bcl-x_S promotes cell death independent of its physical interaction with Bcl-x_L or Bcl-2. It is possible that Bcl-x_S enhances a death signal from a cellular factor induced by the virus or a virus-encoded protein. The finding that the viability of pAdRSV-*lac-z*-infected cells was less than that seen in mock-infected control cells lends considerable support for this notion.

The pattern of DNA degradation and the morphological changes seen in the virally infected cells is consistent with death by apoptosis. Recently, several investigators have proposed that DNA fragmentation in apoptosis occurs in two stages, the first being initiated by a distinct, still-undescribed endonucleolytic activity, which cleaves DNA into 50–300-kb fragments, and a second stage catalyzed by a calcium-magnesium endonuclease (28, 29). Our data are in agreement with these findings and suggest that the virus may induce this primary critical endonucleolytic activity, which appears to be sufficient to cause cell death. These large DNA fragments can be explained on the basis of current knowledge of DNA structure. In both interphase nuclei and metaphase chromosomes, DNA is organized into supercoiled, looped domains of approximately 50–100 kb (30), and DNA sequences are nonrandomly organized in these chromosomal loops, with nontranscribed domains localized to the base of these loops, which are attached to the underlying matrix, called matrix-associated regions (reviewed in Refs. 31–33). These matrix-associated region sequences are A/T rich and contain Topo II sites, making it very likely that Topo II participates in binding of loops to the nuclear matrix (34). The appearance of these large fragments is thought to represent the release of loops of chromosomal DNA from their points of attachment to the underlying matrix and nuclear lamina. The sites and the source(s) of endonucleolytic activity induced by the virus are not known at present, but one can speculate that Topo II or other DNA-binding proteins situated at the base of the loops may be involved.

Our findings indicate that adenoviral expression of Bcl-x_S in NB cells can induce apoptosis even in the context of high-level Bcl-2 and Bcl-x_L expression. Importantly, Bcl-x_S induces an onset of cell death that is rapid and complete. This finding suggests that adenoviral *bcl-x_S* expression may be a useful therapeutic tool in the treatment of patients with NB, possibly as an *ex vivo* purging method prior to autologous bone marrow transplantation. Support for this notion is provided by studies indicating that human hematopoietic progenitor cells capable of repopulating the bone marrow of immunodeficient mice are resistant to killing initiated by the *bcl-x_S* adenovirus (19). Future studies

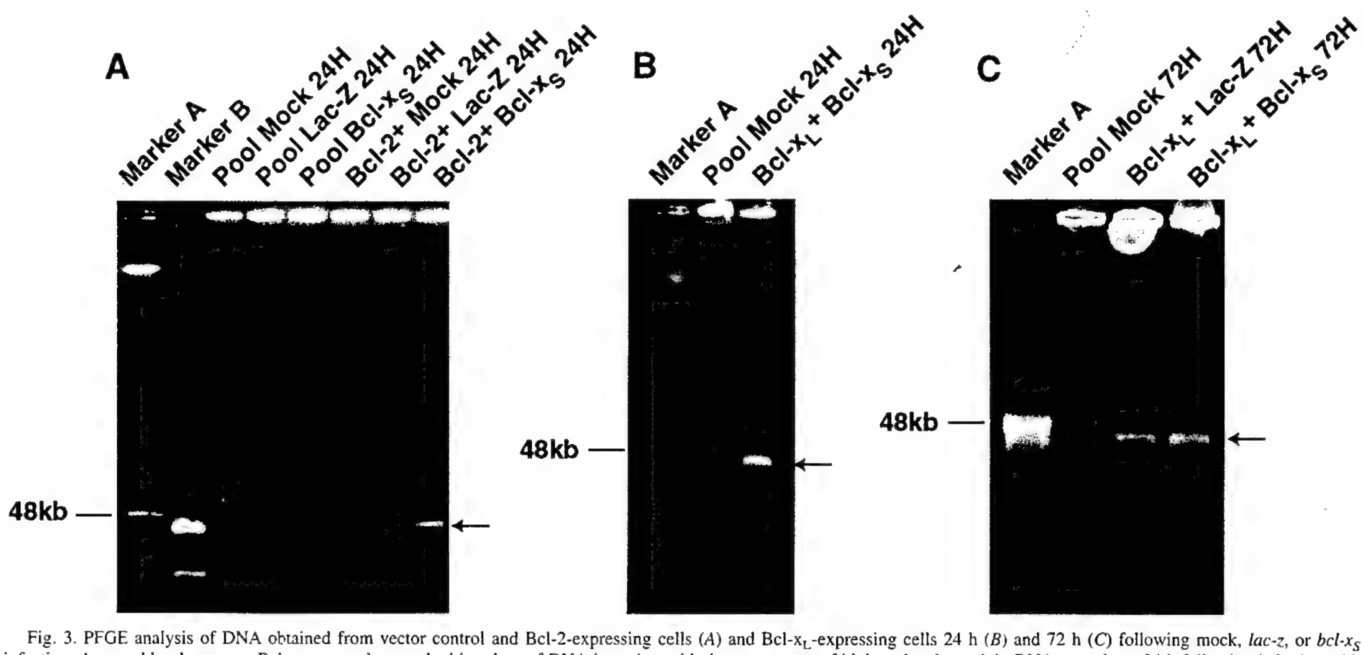

Fig. 3. PFGE analysis of DNA obtained from vector control and Bcl-2-expressing cells (*A*) and Bcl-x_L-expressing cells 24 h (*B*) and 72 h (*C*) following mock, *lac-z*, or *bcl-x_S* infection. As noted by the *arrow*, Bcl-x_S expression resulted in a loss of DNA integrity, with the appearance of high molecular weight DNA as early as 24 h following infection with the *bcl-x* adenovirus. A similar pattern of DNA degradation was seen following infection with the control *lac-z* virus but at a later time point (*C*). *Marker A*, Coliphage λ concatamer DNA ladder (Megabase II; Life Technologies); *Marker B*, λ DNA cut with *Hin*dIII (Life Technologies).

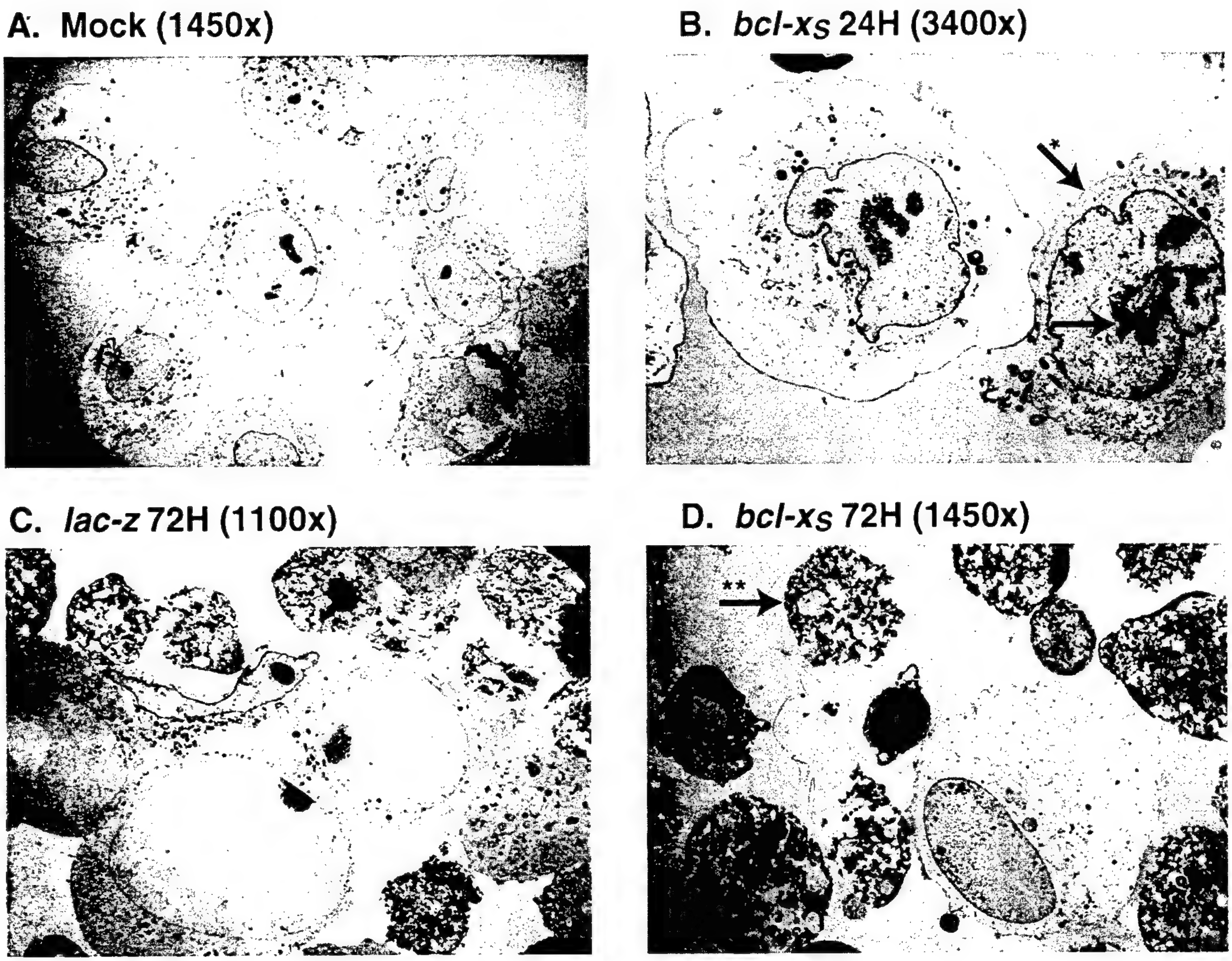

Fig. 4. Representative electron micrographs of SHEP-1 cells 24 and 72 h following infection with *lac-z* or *bcl-x_S* adenovirus. Apoptotic features could be detected as early as 24 h in *bcl-x_S*-infected cells (*B*) but not *lac-z*-infected cells (not shown). By 72 h, the majority of cells infected with either virus (*C* and *D*) showed evidence of apoptosis. *Arrow,* chromatin condensation; * and *arrow,* cytoplasmic retraction; ** and *arrow,* nuclear membrane degeneration.

will need to address this possibility and to determine the precise mechanism by which Bcl-x_S induces DNA damage.

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Targeting cancer cell death with a bcl-x_S adenovirus

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Introduction

Recent advances on the molecular basis of cancer have indicated that transformation is not solely a matter of increased cellular proliferation [60]. It has become widely accepted that more than one genetic alteration is necessary for a cell to become cancerous [80]. This is due to built-in cellular mechanisms that can detect abnormalities and activate genetic programs to remedy them, making single adverse events less likely to cause significant problems. For example, tumor suppressor genes are best known for their ability to halt cell cycle progression and allow time for repair and maintenance of genomic integrity [83]. Malfunction of this system constitutes a second defect that can lead to malignant transformation [80].

More recently, a second protective mechanism known as apoptosis has been identified as important in deleting potentially dangerous cells from an organism [28]. Programmed cell death (PCD) is a genetic program that initiates a series of events resulting in cell suicide. This process leads to apoptosis, a morphologically distinct form of death with physical characteristics such as nuclear fragmentation, membrane blebbing, and DNA degradation [86]. Apoptosis plays a vital role in the normal development of an organism. Its many functions include regression of the tadpole tail in frogs [44] as well as the removal of self reactive lymphocytes in the formation of mammalian immunity [72].

DNA damage and the improper expression of oncogenes have been shown to induce programmed cell death [28]. This is presumably a safeguard against cancer. Genetic defects that permit a cell to constitutively block apoptosis confer a selective growth advantage to that cell. Thus, it makes sense that this is an essential step in the progression to cancer. The discovery and characterization of several apoptosis modulators have verified that this is indeed often the case. Improper regulation of these genes can increase cell survival and provide tumor resistance to traditional forms of cancer treatment (radiation and chemotherapy) that function by activating PCD [32]. This generally leads to poor clinical prognosis.

Fortunately, these same pathways that block cell death represent a potential target for the rational design of new therapies. In this review, we will introduce the factors

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regulating apoptosis in cancer and discuss possible areas for intervention. We will also provide an example of such intervention, in which an adenovirus is used to deliver a pro-apoptotic gene, *bcl-x*$_S$.

Programmed cell death

PCD is best understood in the nematode *Caenorhabditis elegans*, where the fate of all 1090 cells have been tracked. Of these cells 131 undergo cell death during development, and genetic studies revealed the master controls behind this phenomenon. A gain of a function mutant of the *ced*-9 (*ced* for cell death abnormal) gene caused all 131 cells that are normally eliminated to survive, while loss of function *ced*-9 mutations caused more than the usual 131 cells to die [38]. In contrast to *ced*-9, the *ced*-3 and *ced*-4 genes cause cell death [27]. In *ced*-4;*ced*-9 and *ced*-3;*ced*-9 double mutants all cells live, which suggests that Ced-3 and Ced-4 are cell death effectors, and Ced-9, which functions upstream, is an antagonist of these proteins. It is known that Ced-4 binds to Ced-3 resulting in activation of the caspase [42, 85]. Ced-9 binds to Ced-4, blocking its ability to activate Ced-3 [73, 85]. This apoptosis machinery has been well conserved throughout evolution. We now know that Bcl-2 family members are mammalian homologues of Ced-9 [39] and the caspases (or ICE proteases) are homologues of Ced-3 [90]. No homologue of Ced-4 has yet been identified, but the apparent interchangability of these proteins between nematode and mammalian systems has facilitated the ordering of these genes [14, 15, 84].

Improper growth stimulation leads to apoptosis

The expression of key genes is involved in the signal to proliferate. Due to their mitogenic properties many of these are proto-oncogenes and have been implicated in human carcinogenesis, since uncontrolled growth is one of several steps along the path towards cancer. Recent evidence suggests that inappropriate expression of these genes leads not only to proliferation but to activation of the PCD pathway.

Deregulated expression of the c-*myc* gene occurs in up to 30% of human cancers [61]. In normal cells, expression of the c-*myc* proto-oncogene is rapidly up-regulated when a cell initiates proliferation [2]. Although all of the functions of c-Myc are not completely understood, it is a transcription factor that, when highly expressed, is able to overcome growth arrest and to block differentiation [5, 6, 25, 62]. Interestingly, c-Myc, whose expression is so tightly linked with cell growth, has also been shown to induce cell death. When c-Myc is highly expressed in cells that are deprived of growth factor, they undergo apoptosis [29]. Therefore, the consequences of c-Myc expression depends on the context of other proliferative signals.

A similar story holds true for the c-*fos*/*jun* proto-oncogenes. Their protein products associate to form a transcription factor called activator protein-1 (AP-1) [17]. Like c-Myc, AP-1 is induced upon mitogenic stimulation and appears to be involved in mediating cell cycle progression [1]. c-Fos and Jun expression have also been correlated with PCD in response to unfavorable growth conditions or cell injury [10, 23]. Further implicating c-Fos in apoptosis were experiments demonstrating that ectopic expression of c-Fos led to cell death under conditions in which cells were normally quiescent [63]. The converse experiment (inhibition of c-Fos expression) increased cell survival under

conditions which normally led to mass apoptosis [23]. This supports the hypothesis that c-Fos somehow regulates apoptosis.

At first these two opposing functions of both c-Myc and AP-1 may appear to be contradictory. However, closer scrutiny reveals that induction of apoptosis by growth effectors may be an important safety means by which proliferation can be halted if such factors are expressed at inappropriate times. This can selectively eliminate cells with potentially carcinogenic alterations, which often result in proliferation regardless of the external signals provided by their environment. The mechanism by which this takes place is as yet unknown. It is possible that the proto-oncogenes that induce proliferation also inherently produce a continuous death signal that can only be stopped under favorable growth conditions. For example, certain growth factors apparently function to inhibit the cell death pathway [12, 21, 34]. On the other hand, apoptosis may be the result of conflicting growth and quiescence signals within the same cell.

In addition, some tumor suppressor genes also function by regulation of PCD. One of the most common abnormalities in human malignancy is a mutation of the *p53* tumor suppressor gene [79]. Up to 50% of human cancers harbor such mutations. p53 exerts its tumor-suppressing effects in two ways: cell cycle regulation and apoptosis. p53 controls the cell cycle through transcriptional activity. Putative p53 DNA binding sites have been identified and can direct mRNA synthesis upon activation of p53 [43, 64]. Several genes have thus far been shown to be under the direct transcriptional control of p53. One of these, *p21* (also called *waf1* or *cip*-1) is activated by p53 in response to cellular damage [26]. Elevated levels of p21 can cause growth arrest by binding to cyclin-Cdk complexes and inhibiting kinase activity [51]. In addition, p53 has been implicated in the repair of radiation-induced DNA damage and transcriptional repression [49, 70].

Perhaps more importantly, p53 has been shown to induce apoptosis under several conditions. Sometimes the simple restoration of p53 in a transformed cell is enough to cause cell death [89]. In another example, mouse thymocytes lacking p53 are resistant to apoptosis caused by radiation and various forms of chemotherapy, while p53-positive cells die when treated in the same way [18, 52]. This suggests that p53 is a major downstream effector of current methods of cancer treatment.

The ability of p53 to induce apoptosis usually does not require transcription, and some p53 mutants that cannot bind DNA and stimulate RNA synthesis are still able to activate the cell death pathway [36, 82]. Therefore, in some systems the apoptotic function of p53 can be separated from the cell cycle regulation, which does require transcription. However, it should be noted that p53-mediated apoptosis is not always transcription independent. Some studies indicate that p53 promotion of cell death can be transcription dependent [67]. This is further supported by the finding that the Bcl-2 family member (see below) Bax, which is pro-apoptotic, has a promoter containing p53 binding sites and is transcriptionally activated by p53 [55].

Members of the bcl-2 family regulate apoptosis

The *bcl*-2 gene was discovered at the breakpoint of a t(14;18) translocation that commonly occurs in B cell lymphomas [20, 76]. Bcl-2 was shown in culture to suppress apoptosis normally induced by a variety of factors including growth factor withdrawal, γ-irradiation, and chemotherapeutic drugs [58, 69]. The protective effects of Bcl-2 are not universal, as T cell deletion still occurs in the presence of Bcl-2 overexpression

[69]. When constitutively expressed in transgenic mice, Bcl-2 led to an accumulation of B cells [53]. The expansion of the B cell population was not due to enhanced cell proliferation but to decreased cell death. The enhanced B cell life presumably allowed secondary genetic abnormalities to accumulate, and eventually ended in lymphoma. Further investigation demonstrated that Bcl-2 knockout mice were subject to increased apoptosis and loss of mature lymphocytes [78]. Nevertheless, these mice were able to survive through development, implying a functional redundancy with respect to Bcl-2.

During the past few years several homologues of Bcl-2 have been identified. Members of this family of proteins contain one or more of four Bcl-2 homology (BH) regions termed BH1, BH2, BH3, and BH4. Bax, Bad, Bak and Bik negatively regulate apoptosis, apparently by antagonizing Bcl-2 [8, 16, 31, 46, 59, 87]. Another Bcl-2 family member, Bcl-x, can be present in one of two forms depending on how the primary RNA transcript is spliced [7]. The larger of the two Bcl-x proteins, Bcl-x_L, contains all four BH regions and exhibits the highest homology to Bcl-2. In culture Bcl-x_L displays remarkable similarity to Bcl-2 in ability to block apoptotic response to a range of external signals. The smaller protein, Bcl-x_S, contains BH3 and BH4 regions and can actually accelerate apoptosis in certain situations, such as cytokine withdrawal from interleukin-3-dependent cell lines. In addition, Bcl-x_S abrogates the protective functions of both Bcl-2 and Bcl-x_L. The relative levels of Bcl-x_L and Bcl-x_S appears to be an important factor in cell survival.

The actual mechanism by which Bcl-2 family members carry out their actions is an area of intense investigation. The carboxy terminus of Bcl-2 contains a hydrophobic transmembrane domain that localizes Bcl-2 primarily to the outer mitochondrial membrane [48]. Removal of this targeting domain from Bcl-2 and related family members either abolishes or diminishes protective activity, which implies that membrane localization is important for Bcl-2 function [57, 75]. It is likely that Bcl-2 acts by inactivating cell death effectors such as a mammalian version of Ced-4 [15, 84]. In other theories Bcl-2 has been postulated to act by controlling the cytoplasmic level of intercellular species such as p53, Cdks, cytochrome *c*, Ca^{2+}, or reactive oxygen species [3, 41, 47, 50, 54, 66, 88]. How all of these processes relate is still poorly understood.

Apoptosis regulators – who is really in control?

So far we have discussed the c-Myc, AP-1, p53, and Bcl-2 proteins in separate contexts. However, it is obvious that proteins playing such critical roles in the cell must have some degree of interdependence. It is unlikely that there would be so many autonomous apoptosis pathways. A more likely scenario is the existence of multiple ways in which apoptosis can be triggered, all of which converge upon a group of central regulators.

p53 has been shown to activate an apoptosis program not only in response to damage caused by external agents, but also in response to internal cellular dysfunction. This raises the question of whether improper expression of genes such as c-*myc* and *fos*/*jun* induce apoptosis in a p53-dependent fashion. Evidence exists that this is the case. c-Myc-induced apoptosis is not apparent in several cell lines devoid of functional p53, but is restored upon the introduction of wild-type p53 [40, 82]. Similar experiments have shown that this p53 dependence also holds true with c-Fos [63]. Therefore, it appears that p53 acts downstream of Myc and Fos and at least in some cases is an intermediate through which the Myc and Fos cell death signals act.

Bcl-2 and Bcl-x_L can inhibit both c-Myc- and p53-induced apoptosis [4, 30, 33, 68, 77, 81]. This, in addition to the multitude of other death signals that can be antagonized by Bcl-2, suggests that the Bcl-2 family acts downstream of most apoptosis effectors and is one of the final resorts in stopping PCD. Because alteration of Bcl-2 family regulation can block most forms of apoptosis, this represents an efficient manner in which cells could become transformed. Thus, it is logical that Bcl-2 family members might play a role in many cancers. It is currently thought that in up to 60% of all cancers, apoptosis is inhibited through overexpression of a Bcl-2 family member [11, 13, 22, 24, 71].

Therapeutic targeting of apoptosis pathways in transformed cells

Our increased understanding of the hierarchical ordering of apoptosis regulators may be useful in targeting treatment of transformed cells. Since it appears that the majority of cell death pathways converge and are under the control of Bcl-2/Bcl-x_L, negative regulators of Bcl-2 and Bcl-x_L would probably allow a cell to act upon apoptotic signals. The delivery of such a gene to cancerous cells would relieve the protection provided by elevated expression of an apoptosis inhibitor. High expression alone might kill the cells, and lower expression levels could increase cellular sensitivity to radiation or chemotherapy.

To test this hypothesis, our laboratory constructed a recombinant, replication-incompetent adenovirus vector expressing the *bcl-x_S* gene, a functional inhibitor of Bcl-2 and Bcl-x_L [19]. This vector was able to efficiently introduce the gene into a wide variety of cell lines and deliver high levels of expression. As expected, virtually all epithelial-derived transformed cell lines that we have tested to date are killed by the *bcl-x_S* virus via apoptosis. Cancer cells derived from patients with neuroblastoma, kaposi's sarcoma, and breast, colon, ovarian, and head and neck cancers all undergo apoptosis when cells express high levels of Bcl-x_S protein [19]. This is true both for primary cancer cells and established, transformed cell lines. Low level expression of Bcl-x_S sensitizes cells to both chemotherapy [74] and radiation therapy. On the other hand, normal fibroblasts and hematopoietic stem cells are relatively resistant to *bcl-x_S* adenovirus-induced apoptosis.

The use of an adenovirus to deliver *bcl-x_S* has potential clinical utility. Results from our laboratories indicate that resistance of hematopoietic stem cells to the virus is at least in part due to the inability to infect such cells. High-dose chemotherapy and autologous hematopoietic stem cell transplantation is increasingly being used to treat both breast cancer and childhood neuroblastoma [35, 45]. Unfortunately, the autologous stem cells used to rescue the patient from lethal doses of chemotherapy are frequently contaminated with cancer cells [9, 37, 65]. The selective killing of the cancer cells by cytotoxic adenovirus vectors makes such agents ideal for eliminating cancer cells contaminating the stem cells collected for re-infusion. Studies from our laboratories have demonstrated the feasibility of this approach. The *bcl-x_S* adenovirus was able to eliminate 1.5×10^4 cancer cells contaminating 10^6 normal bone marrow cells, whereas the normal human hematopoietic stem cells exposed to the virus were still capable of engrafting the bone marrow of SCID mice [19].

This virus may also be useful for the treatment of cancers in other settings. Because non-replicating viruses will only diffuse for limited distances in solid tissues, they can best be delivered to cells in a cavity. Two diseases in which this virus may be useful

are bladder and ovarian cancer. Early bladder cancer arises in the bladder and ovarian cancer initially spreads in the peritoneal cavity. Furthermore, early bladder cancers are superficial tumors which arise focally or diffusely initially penetrating only a few cell layers of the bladder luminal epithelium. Similarly, early in the course of ovarian cancer, or after initial chemotherapy, there are often microscopic foci of tumor cells remaining. In both of these cases, it is quite possible that all of the cells can be infected and killed by an adenovirus vector.

One area of concern is whether normal, non-transformed cells will be adversely affected by the introduction of Bcl-x_S overexpression. Circumstantial evidence from our laboratory indicates that the virus will preferentially kill transformed cells. This suggests that Bcl-x_S itself does not cause apoptosis. Rather, Bcl-x_S may function by allowing other stimuli to initiate an apoptotic pathway without the interference of Bcl-2 family members. Since transformed cells, by virtue of their genetic lesions, are more likely to deliver these signals than their normal counterparts this may explain the selectively of Bcl-x_S-mediated killing. This mechanism will only become clearer when we better understand the mode of Bcl-x_S action and the role of the Bcl-2 family in survival of normal and transformed cells.

Summary

Transformation is a complex cellular process that requires several genetic abnormalities. In many cases, one of these abnormalities is an inhibition of PCD, which provides a selective advantage for tumor cells. This has been recently shown in an in vivo model, where overexpression of Bcl-x_L is a crucial step in the progression from hyperplasia to neoplasia and is accompanied by a significant decrease in tumor apoptosis [56].

Frequently, overexpression of a member of the Bcl-2 family results in a block in cell death and appears to nullify many built-in cellular defense mechanisms against cancer. Such a block presents a problem because radiation and chemotherapy, standard cancer treatments, ultimately exert their effect by induction of apoptosis and would also be made less effective. Therefore, to better treat cancer it may be necessary to develop novel methods to overcome the effects of the Bcl-2 family. One way to approach this problem is to target the cause – the molecular machinery that allows a cancer cell to survive. Advances in our understanding of apoptosis has identified the Bcl-2 family as a mediator of most apoptosis pathways, including those initiated by oncogenes, tumor suppressor genes, growth factor withdrawal, and external damaging signals. Therefore, functional inhibition of Bcl-2 family members is lethal to many cancer cells. Using gene transfer technology, we can now deliver genes that accomplish this goal. Further investigation will reveal whether this translates to improved therapy in the future.

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A Method of Limited Replication for the Efficient *In Vivo* Delivery of Adenovirus to Cancer Cells

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ABSTRACT

Replication-deficient viral vectors are currently being used in gene transfer strategies to treat cancer cells. Unfortunately, viruses are limited in their ability to diffuse through tissue. This makes it virtually impossible to infect the majority of tumor cells *in vivo* and results in inadequate gene transfer. This problem can be addressed by allowing limited viral replication. Limited viral replication facilitates greater penetration of virions into tissue and can improve gene transfer. We have developed a strategy of limited viral replication using AdRSVlaclys, a chemically modified E1-deleted adenovirus, to codeliver an exogenous plasmid encoding the adenovirus E1 region. This system allows one round of viral replication. We examined the effect of this limited adenovirus replication *in vitro* and *in vivo*. In culture, codelivery of virus and pE1 resulted in a large increase in infected cells when compared with control cells exposed to virus and pUC19. In experiments on nude mice bearing HeLa ascites tumors, intraperitoneal injection of AdRSVlaclys/pE1 resulted in a significantly higher percentage of infected HeLa cells as compared with the PBS controls ($p < 0.05$) or the AdRSVlaclys/pUC19 controls ($p < 0.01$). These data demonstrate that the transcomplementation of replication-deficient adenovirus with exogenous E1 DNA leads to limited replication, and this controlled replication enhances gene transfer efficiency of adenovirus *in vivo*.

OVERVIEW SUMMARY

Replication-defective viral vectors are limited in their ability to diffuse through tissue. This poses a problem for treating tumors *in vivo* using gene transfer. This article demonstrates that limited replication of adenovirus leads to greater gene transfer efficiency *in vitro* and *in vivo* without introducing additional safety concerns beyond traditional adenovirus administration. This has implications for the improvement of current gene transfer methods for treating cancer.

INTRODUCTION

VIRAL VECTORS are among the most efficient vehicles for gene transfer *in vitro* and *in vivo* (Mulligan, 1993). For this reason, replication-deficient viral vectors have been used in various gene transfer approaches to treat cancer cells (Crystal, 1995). Unfortunately, this approach is limited in that it is nearly impossible to infect the majority of tumor cells *in vivo* owing to physical constraints imposed by both the virus and tumor. Tumors *in vivo* are usually present as solid masses or sheets many layers thick, as opposed to the easily infected monolayers in cell culture. Typical virions are large enough to prevent significant diffusion through these cell layers. In addition, in some *in vivo* animal models the amount of virus administered is limited by the volume that can actually be physically injected.

The limitations in vector delivery present a serious problem in targeting cancerous cells *in vivo* using gene transfer. Delivery of a cytotoxic or tumor-suppressing virus may temporarily slow down tumor growth, but is doomed ultimately to fail if some tumor cells are left unharmed. Even strategies with a "bystander effect" (such as the herpes simplex virus thymidine kinase gene) require that a significant amount of tumor cells be infected, and most successful animal models of thymidine kinase delivery have involved the administration of retroviral producer cell lines to ensure that this is the case (Culver *et al.*, 1992; Takamiya *et al.*, 1992; Barba *et al.*, 1994).

It may be possible to circumvent viral delivery problems with controlled replication of a viral vector *in vivo* (Goldsmith,

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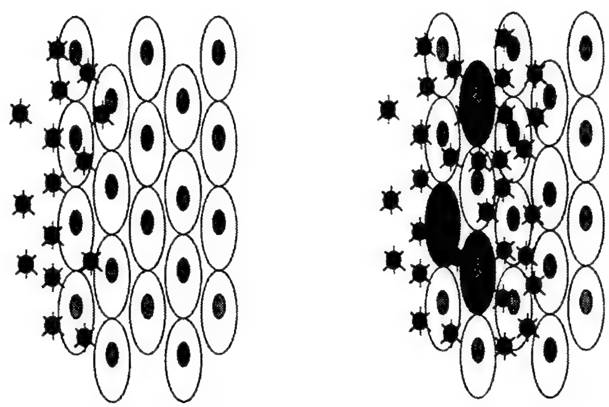

FIG. 1. Schematic diagram of limited replication *in vivo*. On the left, conventional techniques result in gene transfer only near the surface of tissue. On the right, limited replication allows virus diffusion to deeper areas in the tissue. Viral replication is occurring in dark cells.

1994). This could allow greater penetration of the virus beyond the first several cell layers (see Fig. 1). Limited replication would also increase the effective local viral titer. For controlled replication to occur, the genes necessary for viral replication must be codelivered with the viral vector. However, these genes must not be engineered into the viral genome or the result would be a fully replication-competent virus. We have developed a model of controlled *in vivo* replication using adenovirus. Adenovirus is relatively chemically stable and can be manipulated by adding polylysine without completely destroying infectious activity (Wagner *et al.*, 1992; Cristiano *et al.*, 1993; Fisher and Wilson, 1994). This allows exogenous DNA to bind to the modified adenovirus by electrostatic interactions and "piggyback" its way into infected cells. By using this method to codeliver a plasmid encoding the adenovirus E1 region, standard E1-deleted adenovirus can enter a round of replication. We will refer to this method as *limited replication*, because barring recombination events, all of the progeny of this process will be replication deficient, eliminating virus replication after one round. Adenovirus also has other favorable properties for use in this system. Adenovirus can be produced at high titers and infect a broad range of dividing and nondividing cells (Kozarsky and Wilson, 1993). In addition, adenovirus has a lytic life cycle, which lyses the cell after virus replication is complete and thus may expose more tumor surface area for further gene transfer.

Other viral vectors all have one or more deficiencies for use in such a system. Retrovirus integrates into the host genome and will continually produce virus for the life of the cell (assuming the transgene is not lethal). Both of these properties are safety concerns (Varmus, 1988). Retrovirus is also surrounded by a relatively unstable lipid bilayer that is difficult to modify chemically without completely abolishing biologic activity (Rosenberg *et al.*, 1997). Herpes simplex virus has a similar bilayer and presents possible toxicity concerns (Glorioso *et al.*, 1997). Adeno-associated virus (AAV) cannot replicate without adenovirus (Fisher *et al.*, 1997), which defeats the purpose of using AAV in the first place. Therefore, adenovirus appears to be the ideal choice for this system.

Here we demonstrate that transcomplementation of replication-deficient adenovirus with exogenous E1 DNA leads to limited viral replication. We have constructed a simple system in which adenovirus plasmid DNA complexes can be used to achieve this effect *in vitro*. Finally, we have extended this study to demonstrate enhanced gene delivery in a relevant *in vivo* model. This addresses a major problem in current approaches to cancer gene therapy.

MATERIALS AND METHODS

In vitro *limited replication of adenovirus using liposomes*

Construction of AdRSVlacZ has been described (Davidson *et al.*, 1994). The plasmid pE1, which contains the E1A and E1B regions of adenovirus (nucleotides 1–5778), was a gift from E. White (White and Cipriani, 1989). This plasmid, as well as all plasmids described hereafter, was purified by centrifugation through a CsCl–ethidium bromide gradient (Sambrook *et al.*, 1989). HeLa cells (human cervical carcinoma) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% P/S (penicillin [100 international units/ml] and streptomycin [100 μg/ml]). SKOV3 (human ovarian carcinoma) cells were maintained in McCoy's 5A medium containing 10% FBS and 1% P/S.

Cells were seeded in six-well dishes and allowed to reach 80% confluence. Lipofectamine (GIBCO-BRL, Gaithersburg, MD) was used to lipofect either 1 μg of pUC19 or 1 μg of pE1 into each well. Lipofection procedures were done as recommended by the manufacturer. After 24 hr at 37°C, the medium was replaced with 2 ml of fresh medium (2% FBS, 1% P/S) containing 1×10^6 AdRSVlacZ viral particles. Each well was fed with 2 ml of medium (2% FBS, 1% P/S) on day 3 and stained on day 6 or 8 with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). Before staining, cells were fixed in 0.5% glutaraldehyde for 10 min and washed twice with phosphate-buffered saline (PBS)–1 mM $MgCl_2$. Stained cells were examined by light microscopy and photographed.

Preparation of adenovirus/polylysine complex

Polylysine was cross-linked to replication-deficient, recombinant adenovirus using a slightly modified version of a procedure described elsewhere (Fisher and Wilson, 1994). To maleimide-activate the virus, AdRSVlacZ was prepared and concentrated on a CsCl gradient. After centrifugation, this virus was immediately desalted on a 15-ml Sephadex G-50 column equilibrated with PBS, pH 7.0. The viral particle concentration was determined by absorbance at 260 nm, on the assumption that one absorbance unit equals 1×10^{12} viral particles/ml. The titered virus was diluted to approximately 5×10^{12} particles/ml and sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce, Rockford, IL), was added to a final concentration of 25 mM. This reaction was rocked gently at room temperature for 7.5 min, then stopped by adding a one-tenth volume of 1 M Tris, pH 7.0.

While maleimide-activating the adenovirus, a thiol group

was added onto the amino terminus of poly-L-lysine (54 kDa; Sigma, St. Louis, MO) using 2-iminothiolane essentially as described (Fisher and Wilson, 1994). The 2-iminothiolane was removed on a 15-ml Sephadex G-25 column equilibrated with 100 m*M* Tris (pH 7.1 at 4°C), 100 m*M* NaCl, and 2 m*M* EDTA. The first fraction that contained virus as determined by absorbance at 220 nm was saved for further use.

The purified, thiolated polylysine was diluted to a volume equal to that of the maleimide-activated adenovirus mixture using 100 m*M* Tris (pH 7.1 at 4°C), 100 m*M* NaCl, and 2 m*M* EDTA. Then the maleimide-activated virus and modified polylysine were mixed and rocked gently at 4°C for 90 min. The polylysine is cross-linked to the virus at this step. Unreacted maleimide groups were blocked by adding a one-tenth volume of 1 *M* 2-mercaptoethylamine and incubating for 20 min at room temperature. To remove unincorporated polylysine and concentrate the virus, the polylysine-modified virus was then centrifuged on a CsCl gradient. The virus was desalted on a 15-ml Sephadex G-50 column. Storage was in 50 m*M* Tris (pH 7.1), 50 m*M* NaCl, 1 m*M* EDTA, and 50% glycerol at −80°C. This modified virus will be referred to as AdRSVlaclys.

Successful cross-linking was verified by a gel mobility shift assay. In a total volume of 100 μl of PBS, 200 μg of the plasmid pK7GFP (a gift from I. Macara; Casey *et al.*, 1996) was incubated with varying amounts of modified or unmodified virus for 30 min at room temperature. These samples were resolved by gel electrophoresis on a 1.5% agarose gel.

Transfection of exogenous DNA in vitro *using the adenovirus/polylysine complex*

We determined that the amount of cross-linked adenovirus necessary to infect more than 80% of HeLa cells was approximately 1×10^5 viral particles/cell. Therefore, this titer was used for the following infections. AdRSVlacZ or AdRSVlaclys was incubated with or without pK7GFP in a total volume of 100 μl of PBS for 20–30 min at room temperature. HeLa cells at 1×10^5 cells/well in 12-well dishes were washed once with PBS and 0.5 ml of DMEM (2% FBS, 1% P/S) was added to each well. Virus/DNA mixtures were added to their respective wells and the cells were incubated at 37°C overnight. The next day, the medium was replaced with 1 ml of DMEM (10% FBS, 1% P/S) per well. Each experiment was performed in duplicate. One set was trypsinized, fixed, and stained with X-Gal to monitor infection efficiency. The other set was trypsinized, washed once with PBS, and visualized for green fluorescent protein (GFP) using a fluorescence microscope to monitor adenovirus-mediated transfection efficiency. The percentage of infected or transfected cells was determined with a hemacytometer and data represent the mean ± standard deviation of two experiments.

In vitro *limited replication of adenovirus using the adenovirus/polylysine complex*

AdRSVlacZ or AdRSVlaclys was incubated with either 2 μg of pUC19 or 2 μg of pE1 in a total volume of 200 μl of PBS for 20–30 min at room temperature. HeLa or SKNSH cells at 1.5×10^5/well in six-well dishes were washed once with PBS and 1 ml of DMEM (2% FBS, 1% P/S) was added to each well. Virus/DNA mixtures were added to their respective wells and the cells were placed at 37°C overnight. The next day, medium was replaced with 2 ml of DMEM (2% FBS, 1% P/S). Cells were fed on days 3 and 6 with 2 ml of DMEM (2% FBS, 1% P/S). No medium was removed during this time. On day 8 postinfection, the cells were fixed and stained with X-Gal to monitor limited replication of the adenovirus. Staining was either done in the dish and subsequently photographed or done in suspension and the percentage of cells infected was determined with a hemacytometer.

In vivo *limited replication of adenovirus*

Nude mice (CD-1 *nu/nu*; Charles River, Wilmington, MA) were injected intraperitoneally with 1×10^7 HeLa cells. Four days later the mice were injected with a virus/DNA mixture in 100 μl of PBS, using a Hamilton syringe. Each injection contained 7×10^9 particles of AdRSVlaclys with either 2 μg of pUC19 or 2 μg of pE1. Two mice were injected with PBS only. These injections were repeated every other day for a total of four injections.

Eight days after the final injection (to allow time for limited replication) mice were sacrificed. The peritoneal cavity was washed twice with 1.5 ml of PBS and the resulting cell suspension was removed and saved. These samples were centrifuged at $350 \times g$ in a microcentrifuge for 2 min, washed once with PBS, and recentrifuged. The resulting pellets of HeLa and blood cells were resuspended in 1 ml of DMEM (10% FBS, 1% P/S) each and placed in six-well dishes in a total volume of 2 ml of medium. These dishes were cultured at 37°C for 2 days in order to allow most of the blood cells to die or lyse. The dishes were then washed six times with PBS and the remaining HeLa cells were fixed and stained with X-Gal for 48 hr at 37°C. Cells were examined by light microscopy and photographed. The percentage of infected cells was determined for each sample. Groups were compared using a one-sided Student *t* test.

Detection of the adenovirus hexon protein

Twenty-four hours after infection with the chemically modified AdRSVlaclys and 2 μg of either pUC12 or pE1, HeLa cells were harvested and attached to microscope slides. Immunohistochemistry using a fluorescein isothiocyanate (FITC)-conjugated anti-hexon IgG (Chemicon, Temecula, CA) was done using the protocol recommended by the supplier. Microscopy was performed with a Zeiss fluorescence microscope.

RESULTS

Independent codelivery of the pE1 plasmid and adenovirus in vitro

To test whether exogenous E1 plasmid DNA could transcomplement replication-deficient adenovirus *in vitro*, an independent plasmid lipofection was followed by AdRSVlacZ infection of HeLa or SKOV3 cells. A low titer of AdRSVlacZ was used for these experiments in order to make viral replication easily detectable. Over the course of the next 6 to 8 days, cells initially transfected with pE1 showed a substantially

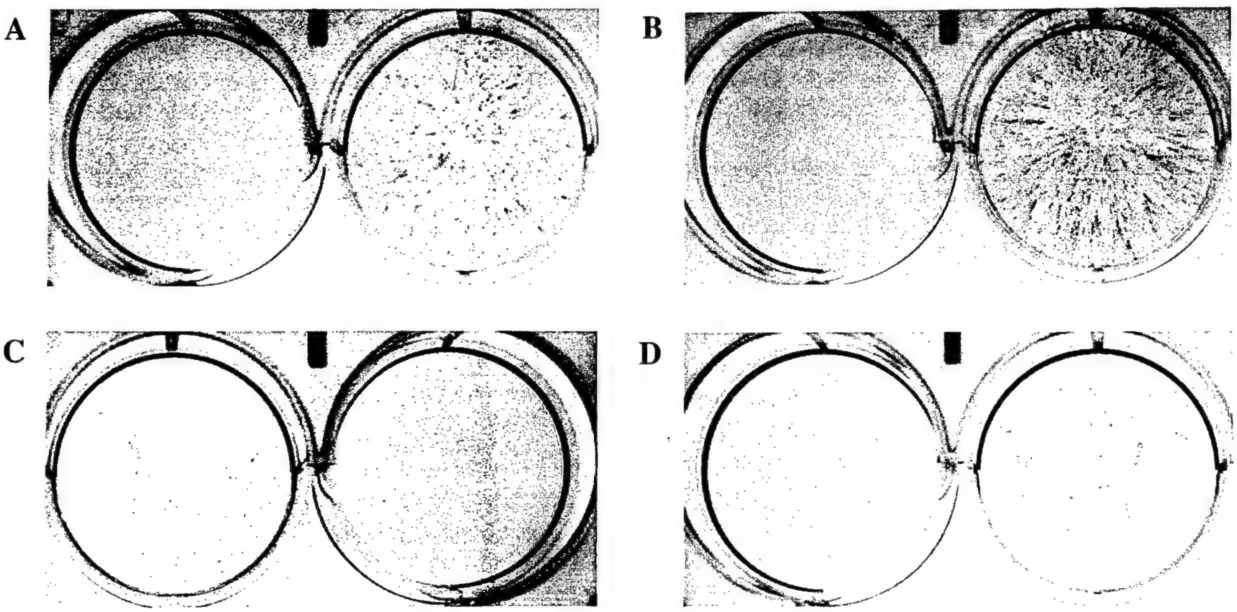

FIG. 2. Lipofection of plasmid DNA followed by adenoviral infection. Cells were lipofected with 1 μg of pUC19 (left) or pE1 DNA (right), then infected with AdRSVlacZ. X-Gal staining is shown at 6 and 8 days. (A) HeLa cells at 6 days; (B) HeLa cells at 8 days; (C) SKOV3 cells at 6 days; (D) SKOV3 cells at 8 days.

greater proportion of infected cells than those initially transfected with the pUC19 control (Fig. 2A). The staining pattern on E1-transfected cells was present in clusters, indicating areas where limited replication and subsequent cell lysis occurred. There was a greater amplification of the adenovirus in the HeLa cells. This may be due to either greater amplification of virus in HeLa cells or greater susceptibility to adenovirus infection of HeLa cells. This verifies our hypothesis that codelivery of pE1 will support viral replication.

Although this shows that the codelivery concept is feasible, the methodology used for these pilot experiments is not practical for general *in vivo* use. Liposomes have been shown to deliver genes *in vivo*, but the efficiency varies dramatically depending on cell type/animal model and would probably need to be extensively optimized for most scenarios (Gao and Huang, 1995). Thus, other methods were explored for the codelivery of pE1.

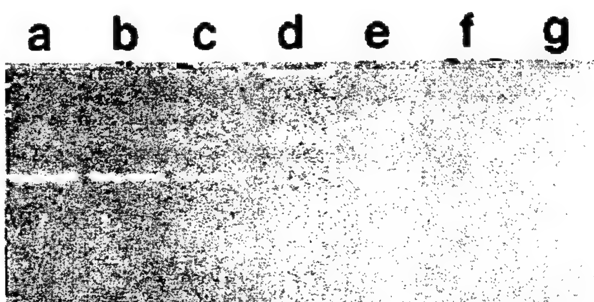

FIG. 3. Gel mobility shift of polylysine-modified adenovirus. AdRSVlaclys, AdRSVlacZ, or no virus was incubated with 200 μg of plasmid DNA. Attachment of DNA to AdRSVlaclys results in a large complex with neutralized charge that does not run into the gel. Lane a, 2.5×10^9 particles of AdRSVlaclys plus DNA; lane b, 5×10^9 particles of AdRSVlaclys plus DNA; lane c, 1×10^{10} particles of AdRSVlaclys plus DNA; lane d, 2.5×10^{10} particles of AdRSBlaclys plus DNA; lane e, 2.5×10^{10} particles of AdRSVlacZ plus DNA; lane f, 2.5×10^{10} particles of AdRSVlacZ (no DNA); land g, DNA only.

Codelivery of plasmid DNA with adenovirus/polylysine complex

Polylysine-modified adenovirus has been shown to complex with and transfect plasmid DNA (Wagner *et al.*, 1992; Cristiano *et al.*, 1993; Fisher and Wilson, 1994). We planned to use such a virus to codeliver the pE1 plasmid. Our method of cross-linking polylysine to AdRSVlacZ was based on the procedure of Fisher and Wilson. This previously reported method essentially modified the entirety of the adenoviral capsid. The modified virus, AdRSVlaclys, would bind to plasmid DNA (Fig. 3).

We aimed next to demonstrate that AdRSVlaclys could transfect plasmid DNA without the assistance of tertiary substrates (such as a polylysine-modified cellular ligand). This would simplify infection procedures for future *in vivo* experiments. Approximately 15% of HeLa cells infected with AdRSVlaclys also introduced the pK7GFP plasmid when examined by fluorescence microscopy (Fig. 4). Because fluorescence microscopy to detect GFP is not terribly sensitive, we expect that the actual percentage of transfected cells is higher. In contrast, cells exposed to plasmid alone or plasmid with AdRSVlacZ exhibited no detectable GFP expression. Thus, the polylysine attached to adenovirus allows codelivery of plasmid DNA.

On the basis of these experiments, we predicted that delivery of the pE1 plasmid complexed with AdRSVlaclys would result in significantly enhanced gene transfer owing to a round

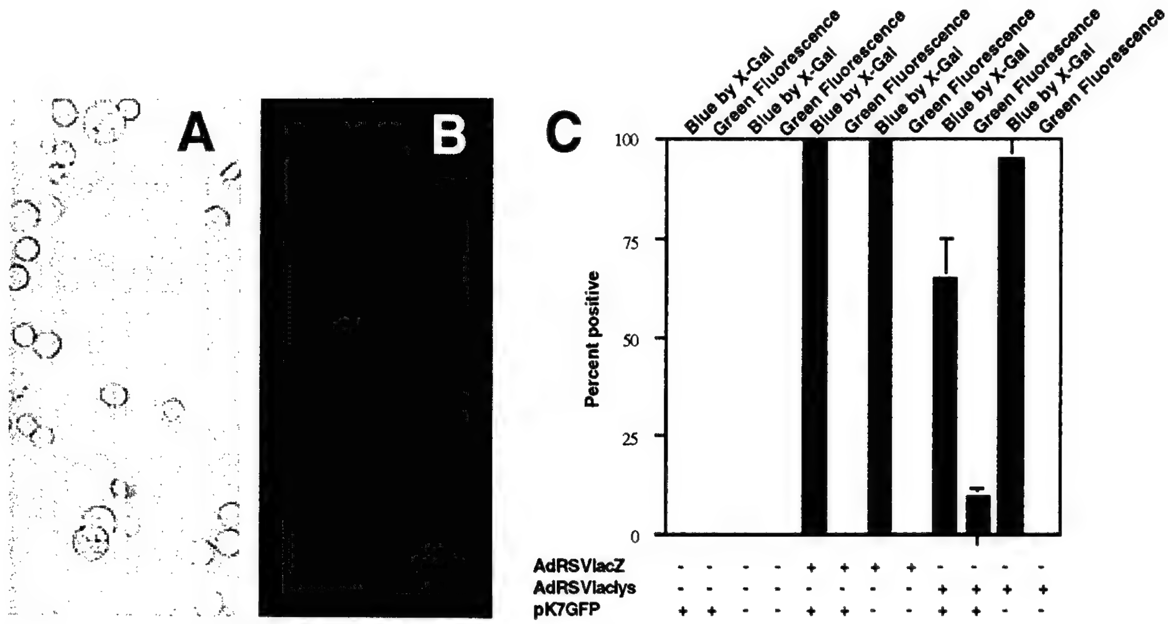

FIG. 4. Transfection of plasmid DNA with AdRSVlaclys. AdRSVlaclys, AdRSVlacZ, or no virus was incubated with pK7GFP before infection of HeLa cells. (A) AdRSVlaclys-infected cells under light; (B) AdRSVlaclys-infected cells using fluorescence microscopy; (C) the infection and transfection efficiencies of various combinations of virus and DNA on HeLa cells. (A and B) magnification: ×200. Addition of plasmid DNA caused a slight decrease in infectivity. This is most likely due to reduced cell membrane binding because of the negatively charged DNA.

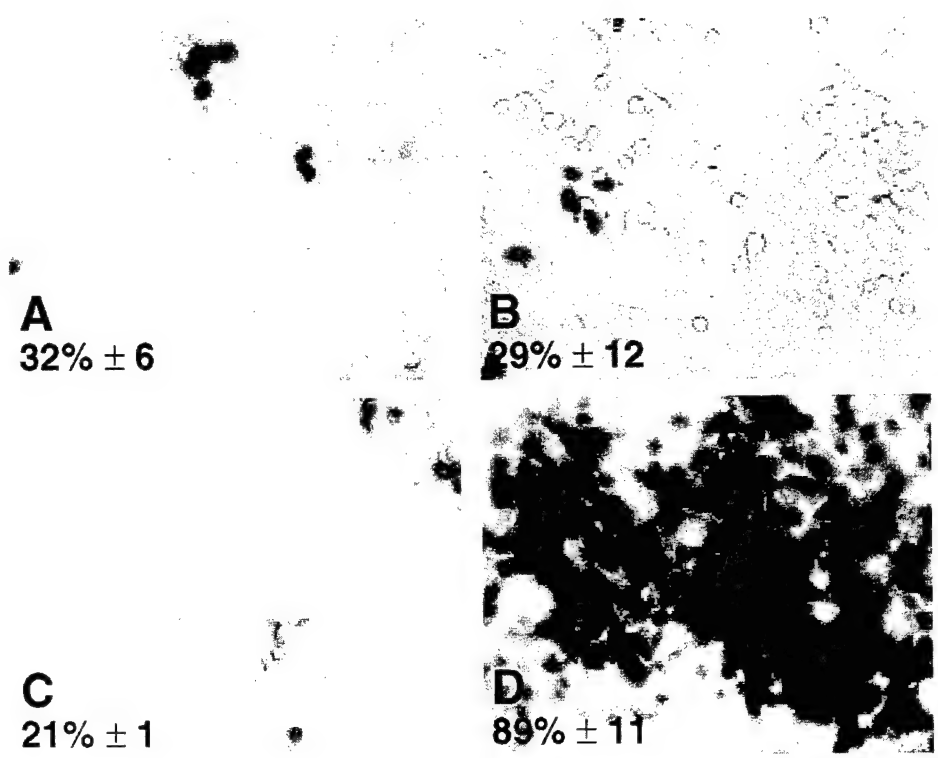

FIG. 5. Limited replication of AdRSVlaclys in HeLa cells. (A) AdRSVlacZ preincubated with pUC19; (B) AdRSVlacZ preincubated with pE1; (C) AdRSVlaclys preincubated with pUC19; (D) AdRSVlaclys preincubated with pE1. Original magnification ×200. Percent infected ± sSD is indicated for (A)–(D).

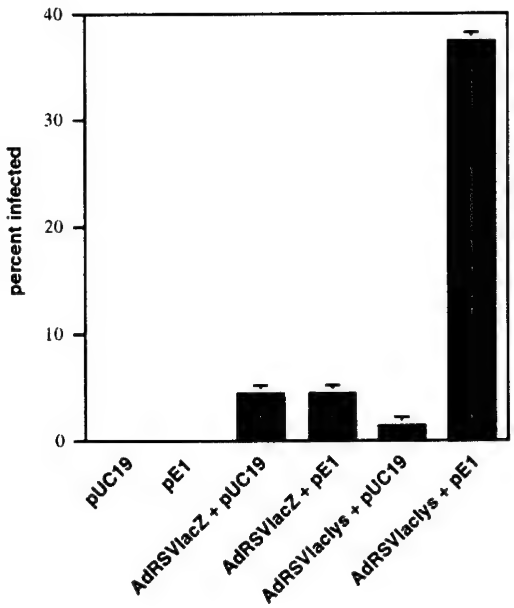

FIG. 6. Limited replication of AdRSVlaclys in SKNSH cells. The experiment described in Fig. 5 was extended to SKNSH cells.

of viral replication. Transfection of only 15% of infected cells translates into a great deal of additional virus when one considers that the typical adenovirus life cycle results in 10^4 viral progeny (Shenk, 1996). When AdRSVlaclys and pE1 were used to infect HeLa cells, a large increase in infected cells was observed by X-Gal staining when compared with the control AdRSVlaclys/pUC19 sample (Fig. 5). Once again, clusters of blue cells indicated areas of viral replication. As expected, infection of HeLa cells with AdRSVlacZ resulted in the same amount of gene transfer regardless of preincubation with pE1 or pUC19. This experiment generated analogous results when repeated with SKNSH (human neuroblastoma) cells, indicating that this effect is not specific to HeLa cells (Fig. 6).

It is possible that the replication observed in AdRSVlaclys/pE1 infected/transfected cells was due to recombination of the replication-defective AdRSVlaclys genome with the transfected pE1 plasmid to generate replication-competent helper virus. This presents a potential safety concern for future *in vivo* applications of this method. To address this concern, the AdRSVlaclys/pE1 infection was repeated on HeLa cells. These infections were harvested at 4 days postinfection, freeze–thawed, and the supernatant of this lysate was used to infect a new monolayer of HeLa cells in a 35-mm dish. These cells were monitored over the next 14 days for the appearance of cytopathic effect (CPE). As a positive control, HeLa cells were incubated with various dilutions of sub360, which is both the original adenovirus strain used to generate AdRSVlacZ (Davidson *et al.*, 1994) and the predicted recombination product of pE1 and AdRSVlaclys. Dilutions ranged from 10^4 viral particles (the amount of virions generated from one viral life cycle) to 10^8 viral particles/well. All dilutions of sub360 produced CPE within the 2-week time period, as evidenced by completely detached, swelled, or lysed cells. Monolayers from HeLa cells cultured with the AdRSVlaclys/pE1 infection extract were intact after 14 days and showed no signs of CPE (data not shown). This strongly suggests that the limited viral replication observed with AdRSVlaclys/pE1 infections is due to transcomplementation, not recombination. This also suggests that helper virus is not a significant contributing factor to any of the results presented in this study.

Enhanced gene transfer in vivo *using limited replication of AdRSVlaclys*

To demonstrate the ability of limited replication to improve transduction efficiency *in vivo*, AdRSVlaclys complexes were injected into mice bearing HeLa ascites tumors. After a series of four injections, the virus was allowed 8 days to replicate, infect more cells, and express β-galactosidase. At the time of sacrifice, there was no apparent toxicity to mice injected with virus. X-Gal staining revealed a low percentage of infected HeLa cells in mice injected with PBS or AdRSVlaclys/pUC19 (data summarized in Table 1). A higher infection percentage was apparent in HeLa cells isolated from mice injected with AdRSVlaclys/pE1 when compared with the PBS group ($p < 0.05$) or the AdRSVlaclys/pUC19 group ($p < 0.01$). Transduction efficiency reached close to 90% in one of the mice (Fig. 7C). *In vivo*/virus replication was confirmed. E1 transfected cells infected with the *lacZ* recombinant adenovirus, but not the pUC19-transfected cells infected with the same recombinant virus, produced the adenovirus late hexon protein (Fig. 8). These data provide evidence that limited replication results in a significantly higher proportion of tumor cells infected *in vivo*.

DISCUSSION

Viral vectors are extremely attractive as gene transfer vehicles owing to their evolved mechanisms for introducing genetic material into cells. This has led to extensive efforts in adapting viruses for the delivery of therapeutic genes (Miller, 1992). In some cases (such as cystic fibrosis), delivery of the therapeutic gene theoretically needs to take place only in a small pro-

TABLE 1. EFFICIENCY OF GENE TRANSFER BY *in Vivo* LIMITED REPLICATION

Mouse number	*Treatment*	*Percent HeLa cells blue*
1	AdRSVlaclys/pUC19	14.0
2	AdRSVlaclys/pUC19	23.0
3	AdRSVlaclys/pUC19	9.4
4	AdRSVlaclys/pUC19	15.3
		15.425 ± 4.89
5	PBS	6.5
6	PBS	3.5
		5 ± 1.5
7	AdRSVlaclys/pE1	21.3
8	AdRSVlaclys/pE1	66.4
9	AdRSVlaclys/pE1	84.6
10	AdRSVlaclys/pE1	69.6
		60.475 ± 23.64

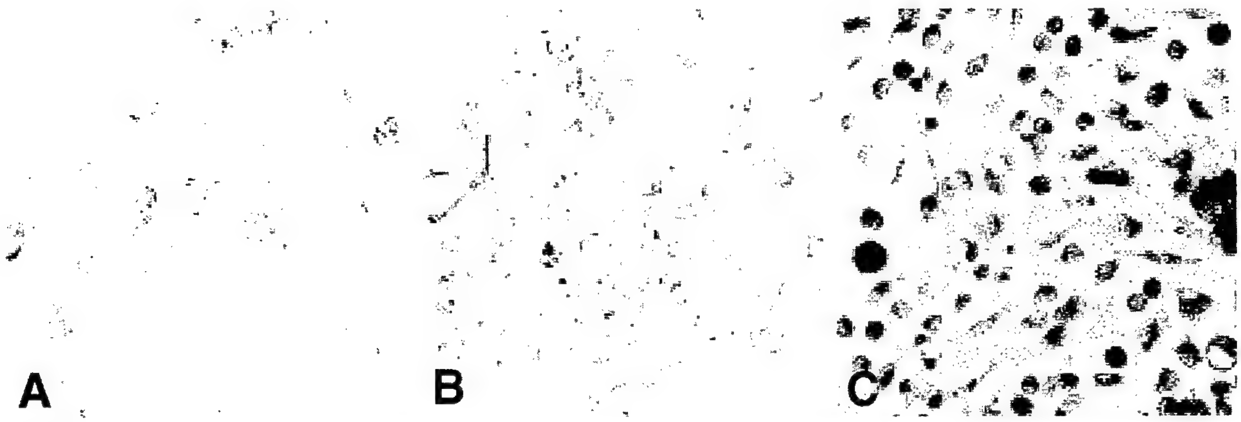

FIG. 7. Examples of limited replication of AdRSVlaclys *in vivo*. Ascites were injected, harvested, and stained as described in Materials and Methods. (A) PBS (mouse 5); (B) AdRSVlaclys with pUC19 (mouse 6); (C) AdRSVlaclys with pE1 (mouse 9). Original magnification: ×200.

portion of affected cells in order to produce significant benefits. However, in other cases, infection of the majority of the target cells may be necessary. For example, the proliferative nature of cancer dictates that it is necessary to infect the majority, if not all, transformed cells in order to achieve true clinical benefit from gene transfer. This problem is exacerbated by the extreme difficulty of transducing the majority of targeted cells *in vivo*, since viruses can diffuse only for limited distances in solid tissues.

Limited viral replication represents a strategy for improving the efficiency of transducing target cells *in vivo*. The feasibility of limited replication has been demonstrated here and by others *in vitro* with several cell lines, using a model system consisting of an E1-deleted adenoviral vector that could introduce exogenous E1 sequences (on a plasmid), permitting replication (Goldsmith *et al*., 1994). Because these DNA sequences were separate from the adenovirus genome and thus were not incorporated into adenoviral progeny, all of the resultant virions were replication defective, ending replication after one round. Minimal helper virus was produced, which indicates that this method may be as safe as traditional adenovirus administration. Adenoviral thymidine kinase-mediated regression of subcutaneous tumors in nude mice arising from cells transfected with E1A *in vitro* was greater than regression of tumors arising from parental cells (Dion *et al*., 1996). We have also extended these results to show that limited replication of adenovirus leads to enhanced gene transfer *in vivo*. The potential of the E1 plasmid with the adenovirus vector to form a replication-competent adenovirus could be further minimized by using an E1 plasmid with no sequences that are shared with the adenovirus vector.

The model system presented in this study is intended only to demonstrate the principle of limited replication and can obviously be improved. Safety concerns regarding the transforming potential of integrated pE1 plasmid sequences can be alleviated by engineering a suicide gene such as the herpes simplex virus thymidine kinase gene into the plasmid. Cells that contain the pE1 plasmid can then be purged from the body, using gancyclovir after the course of treatment. Furthermore, adenovirus cross-linking procedures that result in greater transfection and infection activity have been reported, and would likely lead to better *in vivo* results (Cristiano *et al*., 1993). Ideally, though, chemical modification of the adenovirus capsid would not take place because it invariably decreases infectability. A plausible alternative would have the E1 sequences packaged inside the adenovirus capsid (but not in the genome), which would

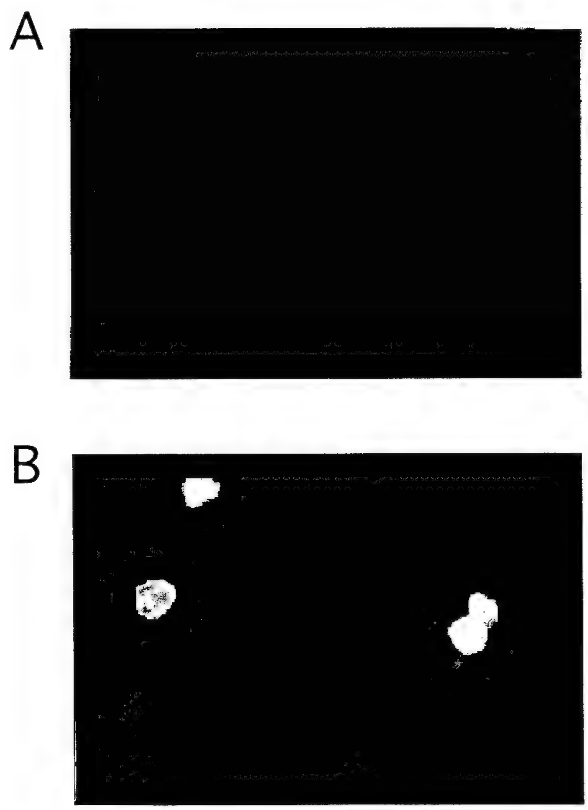

FIG. 8. *In vivo* expression of the adenovirus hexon protein in HeLa cells. Immunohistochemistry showing expression of the adenovirus hexon protein in HeLa cells infected with AdRSVlaclys conjugated with either pUC19 (A) or pE1 (B). Original magnification: ×200.

result in 100% of infected cells undergoing a round of replication, provided the cells are permissive. Current technology does not allow us to do this. Another possible scenario would be to place the E1 sequences in the adenovirus genome, but under tumor-specific promoters. This could be difficult since it is likely that leaky E1 expression would occur owing to viral enhancers present throughout the adenovirus genome (Shenk, 1996).

Limited replication could be especially useful for cancers arising in cavities where adenovirus is easily administered. Two examples of this are bladder and ovarian cancer. Bladder cancers are superficial tumors that arise focally, or diffusely, initially penetrating only a few cell layers of the bladder lumenal epithelium. Ovarian cancer initially spreads in the peritoneal cavity. In both of these cases, it may be possible to infect all transformed cells using limited replication of adenovirus. This would also be a probable improvement on current gene transfer methods in solid tumor masses, although it is still unlikely that all the cells of a large tumor mass can be transduced. In summary, we have shown that limited replication of adenovirus is safe and results in enhanced gene transfer both *in vitro* and *in vivo*. This addresses the problem of inadequate gene transfer in cancer gene therapy and may eventually lead to potential clinical benefits in the future.

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Role of p53 in the Regulation of Irradiation-Induced Apoptosis in Neuroblastoma Cells

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Wild-type p53 plays a crucial role in the control of apoptosis following ionizing radiation (IR); conversely, mutant p53 is associated with IR resistance. Although wild-type p53 is expressed in virtually all neuroblastoma tumors, treatment failures secondary to inadequate local control with radiotherapy are a problem in patients with advanced stage disease. This apparent paradox is the focus of our interest. The Shep-1 neuroblastoma cell line is highly resistant to IR. This cell line contains a wild-type p53 gene and is an ideal model for studying the mechanism of IR resistance in this disease. Following high-dose IR, cell fractionation demonstrated that p53 is induced and targeted to the nucleus. The induced p53 is functional as p53-responsive genes (Waf-1 and MDM-2) are appropriately induced following IR. Intriguingly, overexpression of p53 could reverse the inherent IR resistance of Shep-1 cells. Multiple cell lines expressing variable levels of exogenous temperature-sensitive p53 were generated. Pulse induction of p53 alone did not affect Shep-1 cell viability, while induction of p53, followed by IR, resulted in cell death and DNA fragmentation proportional to the dose of IR and the level of p53 expression. These findings demonstrate that p53 overexpression renders Shep-1 cells IR-sensitive and suggest that large quantities of exogenous p53 can overcome the factors inhibiting p53-mediated, IR-induced apoptosis. © 1998 Academic Press

Key Words: **apoptosis; p53; cellular localization; radiation resistance; neuroblastoma.**

The p53 gene is the most commonly mutated tumor suppressor gene/oncogene found in tumors when considering both sporadic and inherited human malignancies (1,2). The p53 gene is located on chromosome 17 and the protein product is a transcriptional activator that plays a crucial role in the control of cellular differentiation, proliferation, and induction of apoptosis (3–5). It has been hypothesized that p53 functions to alter cell cycle progression to prevent replication of damaged DNA (6). In many cells, apoptosis that follows DNA damage is dependent upon, or enhanced by, the induced expression of wild-type p53. Following exposure to DNA damaging agents, wild-type p53 levels increase 5–60-fold as a result of stabilization of the p53 protein. p53-deficient cells or cells expressing mutant p53 fail to undergo apoptosis in response to DNA damage (6–8). Recent studies have demonstrated that nuclear localization signals are inherent to the primary structure of the p53 protein and that nuclear localization of wild-type p53 is essential for its function as a cell cycle regulator (9). In fact, nuclear exclusion of p53 has been suggested as a mechanism of p53 inactivation in breast cancer (10).

Neuroblastoma (NB)[2] is the most common extracranial malignancy seen in children. The tumor originates in neural crest cells and patients with

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[2] Abbreviations used: NB, neuroblastoma; RSV, rous sarcoma virus; MEM, minimal essential medium; VCI, viable cell index; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, protein agarose gel electrophoresis; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); PFGE, pulse-field gel electrophoresis; IR, ionizing radiation.

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advanced stage disease have a poor outcome despite treatment with aggressive therapeutic regimens involving high-dose chemotherapy and IR treatment (11). Although p53 mutations frequently occur in many CNS tumor types (12) and abnormalities of chromosome 17 are found in primary NB tumors (13), analysis of primary NB tumor specimens has demonstrated p53 mutations in less than 3% of cases (12,14,15). Additionally, the majority of NB cell lines express large quantities of wt p53 protein (16,17). Despite wild-type p53 expression, the functional status of p53 in NB remains controversial. Studies by Moll *et al.* have demonstrated that wild-type p53 protein is localized exclusively to the cytoplasm of undifferentiated NB tumors and cell lines (18,19). It has been suggested that this pattern of expression (i.e., nuclear exclusion) is responsible for a failure to induce G1 arrest following DNA damage (19). Conversely, Goldman *et al.* have found that p53 is induced in NB cells treated with chemotherapeutic drugs. In their system, p53 is detected in nuclear material following treatment and downstream p53 responses are activated (20).

Our laboratory has been investigating the mechanisms contributing to IR resistance in NB. The Shep-1 cell line models the important characteristics of recurrent clinical disease in that this cell type demonstrates IR resistance and expresses wild-type p53. In response to IR, Shep-1 cells were found to induce not only wild-type p53 but also downstream p53 responsive genes (Waf-1, MDM-2). The induced p53 appropriately localized to the nuclear compartment. IR resistance in these cells was not associated with the upregulation of Bcl-2, and Bcl-x_L showed minimal induction beyond basal levels. The absence of apoptosis in the context of an intact p53 response led us to suspect that the mechanism leading to IR resistance involves inhibition of a p53 responsive activity. We tested this hypothesis by determining the potential of p53 overexpression to impart IR sensitivity by overcoming inhibition by a putative competitive inhibitor. Stable transfection experiments produced cells with high levels of p53 protein and an intact apoptotic response to IR.

MATERIALS AND METHODS

Cell culture and transfection. The Shep-1 NB cell line was used for these experiments. This cell line is an epitheloid subclone of the human NB cell line SK-N-SH originally described by Biedler *et al.* (21). The myeloid leukemia cell line ML-1, known to undergo apoptosis in response to IR damage, was used for comparison (7; kindly provided by Dr. M. Kastan, Johns Hopkins Oncology Center, Baltimore, MD). Cells were grown in MEM (Shep-1) or RPMI (ML-1) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin. The expression vector p53^{tshygro} was used to transfect Shep-1 cells. This vector contains the coding sequence of a mouse mutant p53, p53^{val1}-$_{135}$ linked to an RSV LTR promoter. This particular p53 mutant at nonpermissive temperatures (37°C) localizes to the cytoplasm and behaves like mutant protein. At permissive temperature (32.5°C) the p53 protein translocates to the nucleus and attains wild-type conformation (22,23). This vector also contains the hygromycin-resistance gene that enables selection of stably transfected cells using the antibiotic hygromycin. Shep-1 cells were stably transfected with p53^{tshygro} or vector-only DNA (p65^{hygro}) using Lipofectin (Gibco Labs, Grand Island, NY) as described previously (24). Individual hygromycin-resistant clones were isolated and subcultured separately. Stably transfected cells were cultured in 10% MEM containing hygromycin B (Calbiochem, La Jolla, CA) at 300 mg/ml. The vector control represents a pool of cells transfected with p65^{hygro} expression vector.

Sequencing of p53 gene in the Shep-1 NB cell line. The highly conserved region (exon 5 through 8) of the p53 gene that harbors >95% of p53 mutations previously described was sequenced (25,26). Coding portions of the p53 gene were amplified from genomic DNA (12). Oligonucleotides flanking exons 5, 6, 7, and 8 were used for sequencing reactions (12,16). Sequencing was performed using the PRISM Ready Reaction Dye Deoxy terminator sequencing kit (Applied Biosystems, Inc.).

IR treatment of cells and cell viability. Shep-1 NB cells and ML-1 hematopoietic cells were irradiated at the indicated doses in a cobalt irradiator. Following IR, 10^4 cells were plated in triplicate in 96-well microtiter plates. Medium was replaced every other day for the duration of the experiment. Cell viability was assessed using the MTT dye reduction assay (27). For this assay, 125 μl of complete media containing 1 mg/ml MTT was added to each well. Plates were incubated at 37°C for 4 h. Following incubation, cells were lysed in a buffer containing 20% SDS, 50% *N,N*-dimethylformamide (pH of 4.5). Absorbance at 570 nM was determined for each well using an MR 650 96-well multiscanner (Dyna-

tech Labs, Inc.). After subtracting for background absorbance of media, the viability of each cell line was determined as the Viable Cell Index [(VCI) = (A_{570} IR)/(A_{570} IR on Day 0 × 100)]. Data presented are the mean values and standard deviation from triplicate experiments. Similar experiments were performed to determine the viability of p53-transfected cells following IR. For these experiments, cells were irradiated and p53 was pulse-induced by placing cells at permissive temperature (32.5°C) for 8 h. Following p53 induction, cells were returned to 37°C for the duration of the experiment.

Protein analysis. Protein expression (p53, MDM2, Waf-1, Bcl-2, and Bcl-x_L) was determined by Western blotting. Following IR, cells were harvested at indicated times and lysed in a buffer containing 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1 bromphenol blue, and 10% glycerol. Protein was quantitated using a BioRad protein assay kit (BioRad Laboratories, Hercules, CA). Following SDS-PAGE, protein expression was determined using an ECL detection kit (Amersham Life Science, Buckinghamshire, England) and mouse monoclonal antibodies specific for p53, Waf-1, MDM-2 (Ab2, Waf-1, MDM-2 antibodies, Oncogene Science, Carpenteria, CA); Bcl-2 antibody (Pharmingen, San Diego, CA); and Bcl-x_L antibody (Transduction Laboratories, Lexington, KY). Anti-topoisomerase antibody (No. 2012-2, Topogen, Columbus, OH) was used as a control to detect the nuclear protein human topisomerase I in cell fractionation experiments. GAPDH antibody (Chemicon Inc., Temecula, CA) was used as a control for protein loading. Protein expression was quantitated by scanning densitometry using AMBIS Image Acquisition software (AMBIS System, Inc., San Diego, CA).

Immunohistochemical staining. Immunostaining was performed to determine the cellular localization of p53 in cells transfected with the inducible p53 expression vector. For these experiments, vector control cells and p53-transfected cells were plated on Lab Tek slides (Nalge Nunc Int., Naperville, IL) and placed at 37°C or 32.5°C overnight. Cells were fixed and incubated with a mouse monoclonal antibody that recognizes wild-type as well as mutant p53 protein (Ab-1, Oncogene Science, Carpenteria, CA). Antigen antibody complexes were detected using an avidin-biotin peroxidase technique (Vectastain, Vector Lab Inc, Burlingame, CA). Peroxidase activity was detected with the chromogen-3-amino-9-ethyl carbazole that results in a red reaction product.

Cell fractionation. Following IR treatment, cells were washed with PBS, pelleted, and resuspended in HB buffer (Hepes 20 mM, 1 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF). Cells were ruptured with 60 strokes in a dounce homogenizer. Nuclei and cell debris were pelleted at 1500 rpm (750*g*) for 10 minutes at 4°C. Nuclei were enriched by extracting the pellet in 1% NP-40 in Buffer X (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF) for 15 min at 4°C and pelleting at 14,000*g* for 10 min. The postnuclear supernatant was loaded onto a discontinuous sucrose gradient with steps of 10, 30, and 40% in HB buffer with protease inhibitors and centrifuged at 100,000*g* for 2 h at 4°C. Following centrifugation, the cytoplasmic fraction was recovered from the top of the gradient and concentrated using a Centricon 10 microconcentrator (Amicon, Inc., Beverly, MA). The plasma membrane fraction was collected from the 30 to 40% interface, diluted with HB, and pelleted by spinning at 100,000*g* for 45 min. Protein contents from the cytoplasmic, nuclear, and plasma membrane fractions were quantitated and Western blotting was performed as described.

Assessment of DNA integrity by pulse-field gel electrophoresis (PFGE). Day 7 following IR and pulse induction of p53, cells were harvested by trypsinization and pellets were prepared in low melting point agarose as previously described (28,29). Cell pellets were loaded into wells in a 1.2% agarose gel and run at 6 V/cm with ramped switch time of 26 s over 16 h using the Chef DR-III System apparatus (BioRad, Richmond, CA). Gels were stained in ethidium bromide and destained in distilled water. *Coliphage* DNA concatamers (Megabase II, BRL) were used for size standards.

FACS analysis to assess DNA damage. FACS analysis was used to assess DNA damage following IR in control and p53-transfected cells. For these experiments, cells were plated in triplicate at a density of 10^5 cells per well in 6-well plates and were irradiated at a dose of 25 Gy. Following IR, p53 was pulse-induced by placing the cells at permissive temperature for 8 h. Following incubation, cells were harvested by trypsinization, suspended in hypotonic lysing buffer (0.1% sodium citrate, 0.01% Triton X-100, and 0.1 mg/ml of propidium iodide), and incubated for 4 h at 4°C (30). Nuclei were analyzed for DNA content on FACS (Becton Dickinson, CA) using Lysis II software. The Sub G_0 fraction (representing apoptotic nuclei with degraded DNA) was deter-

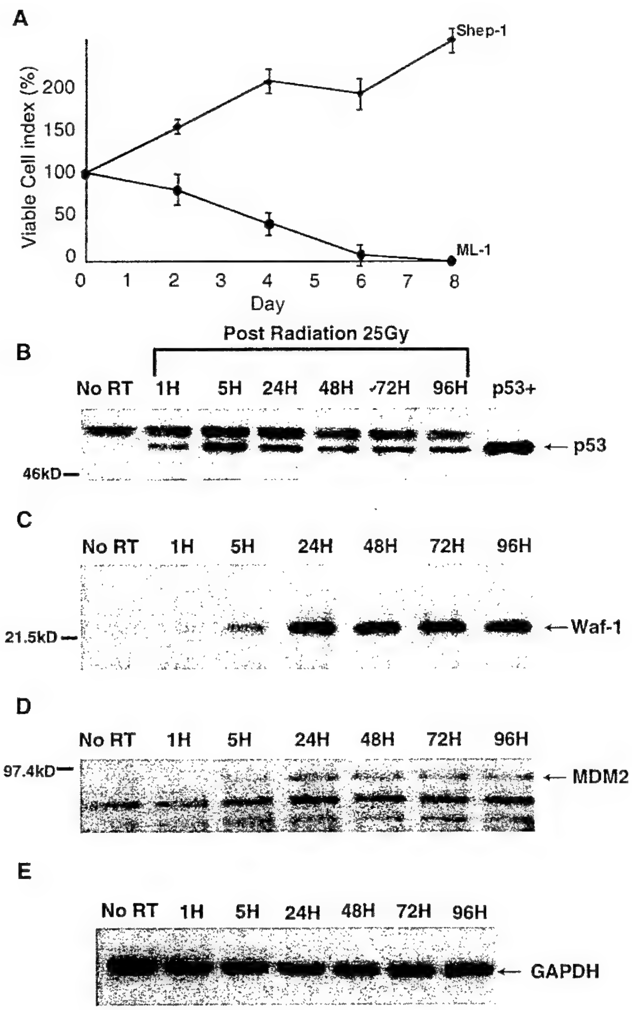

FIG. 1. (A) MTT viability assay of Shep-1 NB cells and ML-1 hematopoietic cells following 25 Gy IR. Shep-1 cells sustain viability and continue to proliferate following high-dose IR. Western blot for (B) p53, (C) Waf-1, (D) MDM-2, and (E) GAPDH, in Shep-1 cells following IR at 25 Gy. p53 was detected as early as 1 h, was maximal at 5 h, and showed sustained expression 96 h following IR. Waf-1 and MDM-2 were both induced at 5 h, showed maximal expression at 24 h, and sustained expression to 96 h following IR. GAPDH was used to control for protein loading.

mined. Differences in the percentage of DNA fragmentation between vector control and p53-expressing cells were determined using Student's paired t test.

RESULTS

Shep-1 NB cells are highly resistant to IR-induced apoptosis. Shep-1 cell viability following IR was determined by the MTT assay. As shown in Fig. 1A, Shep-1 cells remained viable following treatment with 25 Gy IR. By way of comparison, the ML-1 hematopoeitic cell line showed a rapid loss of viability over the same time period (VCI Day 8, 250% Shep-1 vs 0% ML-1, $P = 0.001$).

Shep-1 cells express wild-type p53 that is normally regulated after IR. Direct sequencing of the p53 gene in Shep-1 cells failed to identify any mutations between exons 5 and 8, the region which harbors the majority of p53 mutations previously described (25,26) (data not shown). Western blot analysis was used to determine the kinetics of p53 expression following IR treatment. As shown in Fig. 1B, p53 was induced in Shep-1 cells by 1 h, maximally expressed at 5 h, and showed sustained expression through 96 h following IR. Additionally, the p53 downstream targets, Waf-1 and MDM-2, were similarly induced following IR. Waf-1 was detected at 5 h, maximal at 24 h, and showed sustained expression through 96 h following IR (Fig. 1C). MDM-2 showed low basal expression pre-IR and was induced by 24 h with sustained expression through 96 h following IR (Fig. 1D).

p53 and Waf-1 appropriately localize to the nucleus following IR. Wild-type p53 affects the cell cycle only when it is localized to the nuclear compartment (9). In order to determine whether Shep-1 cells fail to undergo IR-induced apoptosis as a result of inappropriate cytoplasmic sequestration of p53, cell fractionation analysis was performed. As shown in Fig. 2A, prior to IR, a small amount of p53 was detected in the whole cell lysate and the nuclear fraction. p53 could not, however, be detected in the plasma membrane or cytoplasmic fractions. Two hours following IR, p53 was dramatically increased in the whole cell lysate and cell fractionation demonstrated that virtually all p53 was present in the nuclear fraction. Waf-1 (Fig. 2B) is localized to the cytoplasm prior to IR. Following IR, Waf-1 expression increases and demonstrates both cytoplasmic and nuclear localization. The nuclear protein topoisomerase I was used as a control for these experiments and is only detected in the nuclear fraction (Fig. 2C). These results convincingly argue that cytoplasmic sequestration of p53 is not the basis for IR resistance in Shep-1 cells.

Deregulated overexpression of p53 in Shep-1 cells. Working on the suspicion that p53 function is inhibited, potentially by a competitive mechanism, we proceeded with experiments to determine if p53 overexpression would overcome the resistance mechanism operating in Shep-1 cells. Toward this

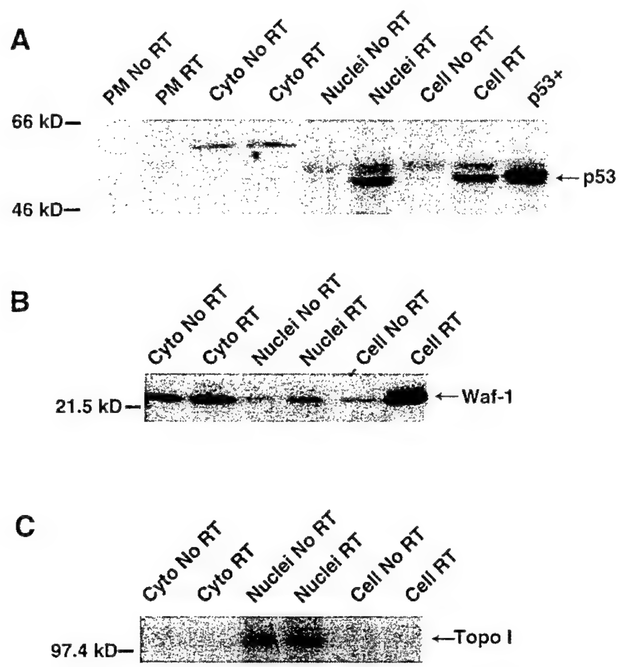

FIG. 2. Cell fractionation for (A) p53, (B) Waf-1, and (C) topoisomerase I, in Shep-1 cells following 25 Gy IR. p53 was induced in the whole cell lysate and localized to the nucleus. Waf-1 was present pre-IR (No RT) in the cytoplasm and post-IR (RT) localized to both the cytoplasm and the nuclear fractions. Topoisomerase I was used as a control and was detected only in the nuclear fraction pre and post-IR.

end, Shep-1 cells were transfected with an inducible temperature-sensitive p53 expression vector ($p53^{tshygro}$). A total of eight hygromycin-resistant clones was isolated. As shown in Fig. 3A, five of the cell lines expressed variable levels of p53 protein. Two cell lines expressing high levels of p53 (clone 5 and clone 7) were chosen for subsequent experiments.

Immunohistochemical analysis demonstrated nuclear translocation of p53 protein in the transfected cell lines. For these experiments, a p53 antibody that recognizes both wild-type and mutant protein was used. As shown in Fig. 3B (panel a), a small amount of endogenous p53 could be detected by immunostaining in vector control cells and this staining pattern was not affected by temperature shift (panel b). In contrast, the p53-transfected clone 5 expresses high levels of immunostainable p53 protein which is cytoplasmic in location at the nonpermissive temperature (37°C, panel c), but shifts to the nucleus when the temperature is dropped to 32.5°C (panel d). To determine the relative level of p53 protein in transfected cells as compared to vector control cells, cells were irradiated at 25 Gy and p53 was pulse-induced by placing cells at permissive temperature for 8 h. p53 protein was detected by Western blotting and was quantitated by scanning densitometry. As shown in Fig. 3C, following IR and pulse induction of p53, clone 5 cells produce 15 times and clone 7 cells 22 times the level of p53 found in irradiated control cells.

p53 overexpression following IR induces apoptosis in Shep-1 cells. The p53-transfected cell lines were used to determine whether deregulated expression of p53 could increase the sensitivity of Shep-1 cells to IR-induced apoptosis. Vector control cells, like parental cells, remained viable following exposure to 5, 10, and 25 Gy IR (data not shown). Pulse induction of p53 without IR had no effect on the viability of clone 5 or clone 7 cells (Fig. 4A). Additionally, clone 5 and clone 7 cells maintained their viability following 5 Gy IR and pulse induction of p53 (Fig. 4B). In contrast, pulse induction of p53 at the permissive temperature and IR with 10 or 25 Gy resulted in rapid and complete loss of cell viability. This loss of viability was dependent on the level of p53 expression. As shown in Fig. 4C, clone 5 cells were able to sustain viability for 5 days following 25 Gy IR. In contrast, clone 7 cells, which express the highest level of p53, showed loss of viability as early as 24 h following IR. Overexpression of p53 following IR results in a loss of viability in Shep-1 cells that is dependent on the dose of IR and the level of p53 overexpression.

p53 overexpression restores apoptotic response to IR. Apoptosis in NB cells is marked by high-molecular-weight DNA degradation. Therefore, to determine if the decrease in viability was mediated by apoptosis, FACS and PFGE were used to assess DNA integrity following IR. As shown in Fig. 5, following IR at 25 Gy the vector-transfected control cells fail to show any evidence of DNA fragmentation. Similarly, the p53-transfected cell lines also maintain DNA integrity following pulse induction of p53 *without* IR. Following pulse induction *with* IR, clone 5 and clone 7 cells demonstrate large-fragment DNA degradation with the appearance of a discrete 50-kb fragment, characteristic of apoptosis in NB cells (28–30). Apoptotic changes were confirmed and quantified by FACS analysis of PI-stained nuclei from similarly treated cells. Six days following pulse induction of p53 and treatment with 25 Gy IR, the majority of clone 7 cells were apoptotic in contradiction to vector-transfected controls (78 ± 1.7% clone 7

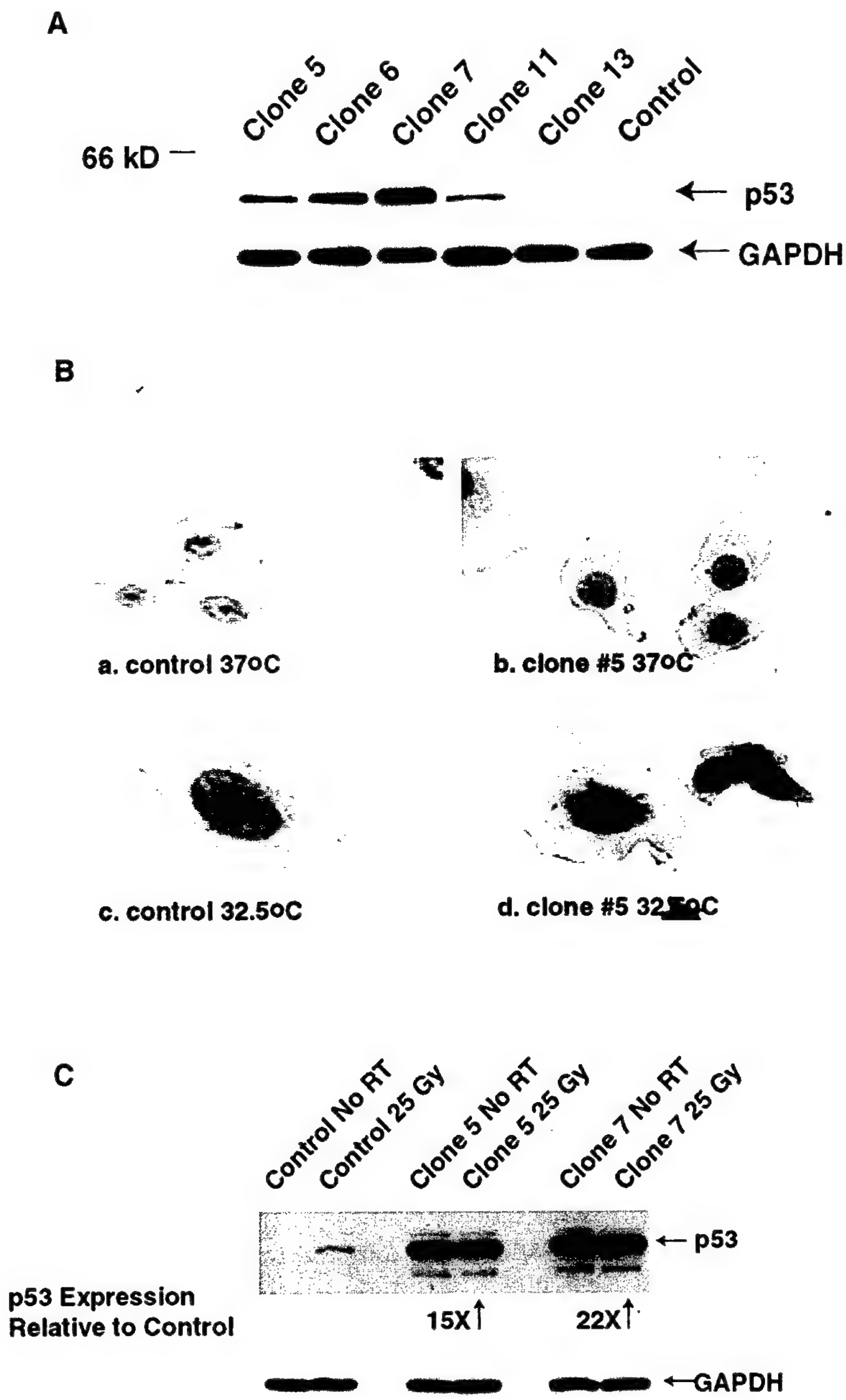

FIG. 3. (A) Shep-1 cells were transfected with an inducible temperature-sensitive p53 expression vector ($p53^{tshygro}$) or vector only (p65hygro). Expression of p53 was determined by Western blot. The transfected cells expressed variable levels of p53 protein. GAPDH expression was used to control for protein loading. (B) p53 immunolocalization in transfected cells was determined by immunostaining. Control cells expressed low levels of immunodetectable protein at both 37 and 32.5°C (panels a and c). At nonpermissive temperatures (37°C) p53 localizes to the cytoplasm in clone 5 cells (panel b). At permissive temperature the p53 translocates to the nucleus as indicated by the bright red nuclear staining (panel d). (C) p53 expression following 25 Gy IR and pulse induction of p53 at permissive (32.5°C) temperature. p53 induction in transfected cell lines was compared to control lines after IR to determine the degree of p53 induction beyond control levels. Clone 5 expresses 15 times and Clone 7 22 times more p53 than the vector-transfected control cells after IR treatment. GAPDH was used to control for protein loading.

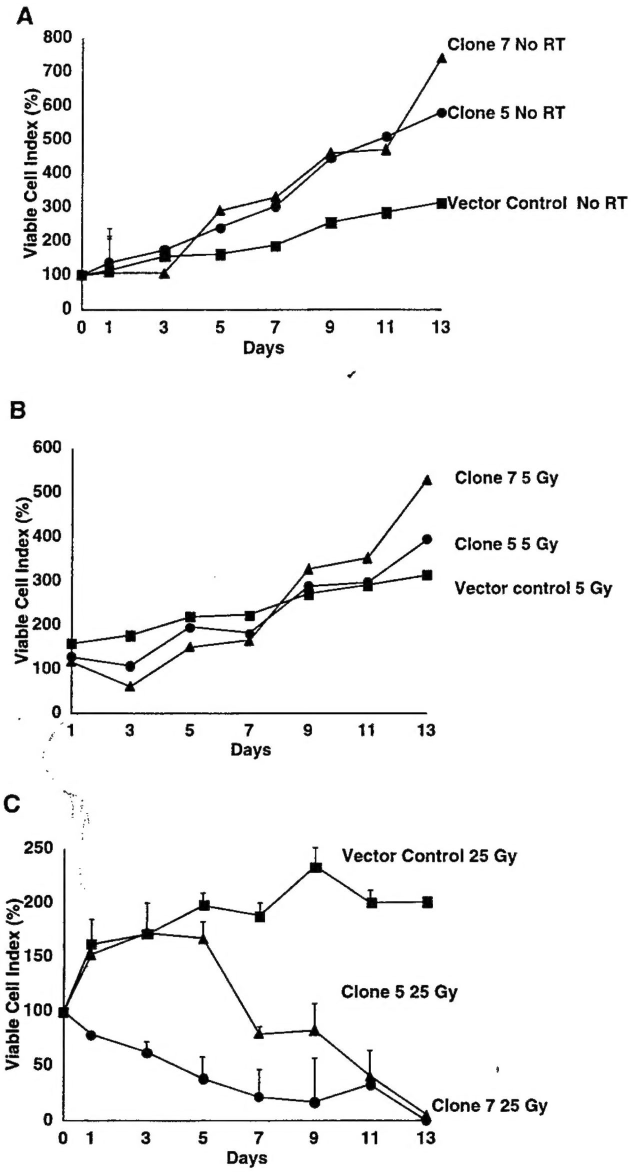

FIG. 4. Cell viability of vector control cells (■), clone 5 (▲), clone 7 (●) cells following pulse induction of p53 at permissive temperatures without IR (4A), with 5 Gy IR (4B), and with 25 Gy IR (4C). Pulse induction of p53 alone or with 5 Gy IR does not result in loss of viability in control or p53-transfected cells. Following 25 Gy IR and induction of p53, clone 5 and clone 7 cells show a rapid loss of viability that is dependent upon the level of p53 expression.

vs 2.4 ± 0.5% controls, $P = 0.001$). These results are consistent with the cell viability data and suggest that overexpression of p53 following IR restores the ability of Shep-1 cells to undergo apoptosis in response to gamma radiation. These data are consistent with the hypothesis that an inhibitory factor is mediating IR resistance in these cells.

Bcl-2 and Bcl-x_L expression following IR. The negative regulators of apoptosis, Bcl-2 and Bcl-x_L, both inhibit p53-dependent IR-induced apoptosis in other cell types (31,32). As such, high-level expression of these proteins could explain the IR-resistant phenotype of Shep-1 cells. This possibility was explored by determining the expression of these genes in our system. As shown in Fig. 6A, Bcl-2 is not expressed in Shep-1 cells prior to, or following, IR treatment at the level of detection afforded by Western analysis. Bcl-x_L is expressed at a low basal level and is induced 1.4 times basal levels following IR (Fig. 6B). It is unlikely that Bcl-x_L expression is inhibiting p53-dependent IR in Shep-1 cells as increases of this magnitude have been found to be inconsequential in determining cell survival following IR in other systems (33).

DISCUSSION

Treatment failures secondary to ineffective local control occur commonly in cases of NB, especially in older children who present with advanced stage disease (34). Although significant advances have been made in the study of chemotherapy resistance in NB, less is known about the factors contributing to IR resistance in this disease. IR controls malignant behavior by both inhibiting growth and killing tumor cells. Tumor cell death induced by IR occurs through an active cellular process involving a number of genes and proteins expressed in direct response to DNA damage (35). p53 is induced follow-

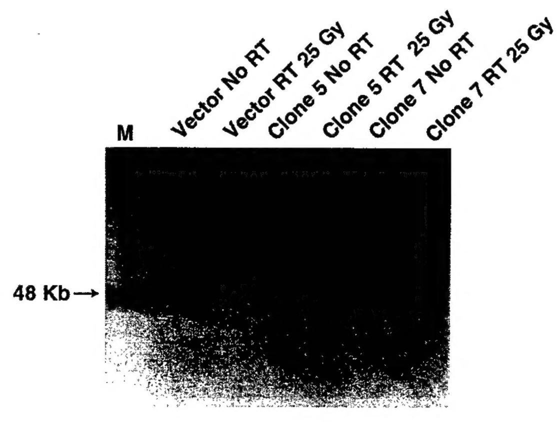

FIG. 5. Western blot of Bcl-2 and Bcl-x_L expression in Shep-1 cells following 25 Gy radiation. Bcl-2 is not detectable in Shep-1 cells pre- or post-IR. Low levels of Bcl-x_L protein are detectable in pre-IR samples and a small increase in Bcl-x_L expression occurs following IR.

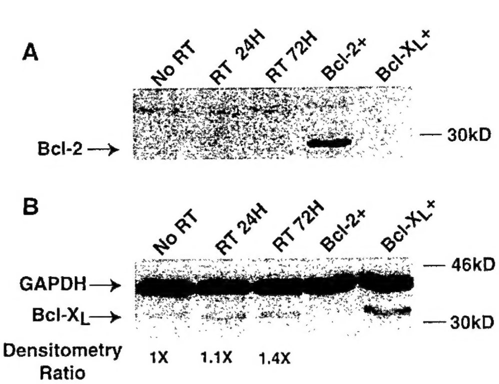

FIG. 6. DNA integrity following 25 Gy IR and pulse induction of p53. Clone 5 and Clone 7 cells show loss of DNA integrity with the appearance of fragmented DNA migrating at ~50 kB. *Coliphage* Lambda DNA concatamers are used as markers. (A) Clone 5 cells proliferate at the same rate as vector control cells until Day 5 when they develop rapid loss of viability. (B) Clone 7 cells, expending the highest level of induced p53, show a rapid loss of viability.

ing gamma radiation and its expression is associated with suppression of growth and induction of apoptosis. As an example, wild-type p53 expression is necessary for murine thymocytes to undergo apoptosis in response to IR (36,37). Supporting the clinical importance of p53 in determining IR response are observations on the phenotype of cells derived from patients with Li-Fraumeni syndrome carrying a germline p53 mutation and studies from p53-deficient transgenic mice. In both systems, loss of p53 function results in IR resistance (38–41).

Although the Shep-1 NB cell type is similarly resistant to high levels of IR, the p53 gene in these cells has wild-type sequence in exons 5 through 8. We limited our sequence analysis to this region of the p53 gene as it is the portion conserved across evolution and because this region harbors 95–98% of known mutations (25,26). It is exceedingly unlikely that mutations outside this region, if present, could account for this resistant phenotype.

Following IR treatment, increased levels of p53 protein were detected in Shep-1 cells following a time course of induction similar to that observed in other IR-sensitive models (6–8). Hence, a failure to increase p53 protein levels is not occurring. Important studies on neuroblastoma by Moll *et al.* (10) have suggested that cytoplasmic retention of p53 in some NB cells results in inappropriate cell survival, consistent with activities of p53 that depend on nuclear localization. However, carefully controlled cell fractionation convincingly demonstrates that in Shep-1 cells p53 induction is accompanied by nuclear translocation. Moreover, the p53 responsive genes Waf-1 and MDM-2 are induced as well, suggesting intact p53 transcriptional activity. Thus, Shep-1 cells contain an intact wild-type p53 response to IR yet demonstrate extreme IR resistance suggesting intact p53 transcriptional activity. The Shep-1 cell line represents the S, or substrate-adherent type of NB cell line (42). Recent work by Isaacs *et al.,* demonstrated that p53 in S-type NB cells is biologically functional following DNA damage (43). Our results support their findings as Shep-1 cells induce p53 following IR that functionally localized to the nucleus and induce downstream responsive elements, yet Shep-1 cells fail to undergo apoptosis. Thus Shep-1 cells contain an intact wild-type p53 response to IR yet demonstrate extreme IR resistance. It must be determined whether this is a common feature of S-type cells or merely a characteristic unique to the Shep-1 cell line.

The ineffectiveness of the p53 response to induce apoptosis in Shep-1 cells could be explained by several possibilities. We speculated that the two most likely reasons were: (1) Shep-1 cells lacked a necessary function that allows apoptosis to occur once p53 is activated, or (2) Shep-1 cells contained high levels of an inhibitory factor specifically interacting with the death response to p53 as transcriptional activities were intact. The two alternative hypotheses were tested by overexpressing p53. If the downstream death mechanism were deficient, overexpression would not overcome IR; however, it could overcome a process of competitive inhibition. Shep-1 cells were transfected with a temperature-inducible p53 expression vector. p53-transfected Shep-1 cells expressing large quantities of exogenous p53 underwent IR-induced cell death as evidenced by a loss of cell viability and DNA integrity following IR and pulse induction of p53. Importantly, the pulse induction of p53 alone without IR did not result in apoptosis of transfected cells. Furthermore, successful induction of apoptosis was dependent on both the dose of IR and the level of p53 expression. These results suggest that the effectors of the apoptotic response to severe DNA damage induced by IR are intact in Shep-1 cells and that overexpression of p53 can reverse the resistant phenotype.

An explanation of the IR resistance in Shep-1 cells is most consistent with the presence of inhibitors to p53's apoptotic response. This mechanism can apparently be overcome by increasing the effective p53 concentration in transfected cells. There are many

factors that regulate p53-induced apoptosis, including the Bcl-2-related gene products (31–33). However, in Shep-1 cells, it is unlikely that Bcl-2 or Bcl-x_L contributed to the IR resistance as Bcl-2 was not expressed and Bcl-x_L showed only minimal post-IR induction. Given the presence of some Bcl-x_L induction, it is possible that other anti-apoptotic genes in this growing family of proteins may contribute to this resistance. It is also possible that the IR seen in Shep-1 cells is a result of a failure to cleave Bcl-x_L releasing the 16 kDa proapoptotic BH3 domain (44). Other gene products that interact with p53, including A20 (45) and p53CP (46) could also be involved in the resistance mechanism. Future studies will need to address these possibilities.

In summary, the Shep-1 system provides an excellent model for identifying putative inhibitors of p53 function. Our findings provide a rational basis for further pursuing clinical strategies to deliver p53-expressing vectors to tumor tissue. The results suggest that even in the context of normal p53 expression, gene therapy aimed at overexpressing p53 in NB tumors could enhance the effectiveness of conventional IR therapy.

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